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REGULATION OF ETHYLENE RECEPTOR EXPRESSION IN *ARABIDOPSIS*

by

Xue-Chu Zhao
B. ENG. , East China University of Science and Technology, 1991

DISSERTATION

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirement for the Degree of

Doctor of Philosophy

in

Biochemistry

September, 2002

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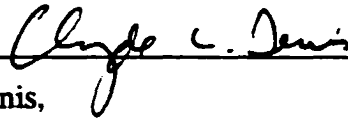

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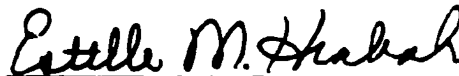

Estelle M. Hrabak,
Associate Professor of Plant Biology and Genetics

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I dedicate this dissertation to my husband Junji, who is always there for me. I also dedicate this dissertation to my son William, whose arrival to this world is the best reward for all my hard work and who also initiates another dissertation for me to work on forever.

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ABSTRACT

REGULATION OF ETHYLENE RECEPTOR EXPRESSION IN *ARABIDOPSIS*

by

Xue-Chu Zhao
University of New Hampshire, September, 2002

The ethylene receptor family of *Arabidopsis* consists of five members, ETR1, ETR2, ERS1, ERS2, and EIN4. Regulation of ethylene receptor expression in *Arabidopsis* was studied using three different approaches. First, the effect of ethylene-pathway mutations upon expression of *ETR1* was examined. Ethylene-insensitive mutations in *ETR1* resulted in a post-transcriptional increase in levels of the mutant receptor. Treatment of seedlings with silver, which leads to ethylene insensitivity, also resulted in an increase in levels of ETR1. Mutations in other components of the ethylene pathway had little effect upon expression of *ETR1*. Second, microarray analysis was performed using reciprocal mutants to examine ethylene-regulated gene expression. A large subset of ethylene-induced genes were involved in ethylene signal transduction. Among these, the ethylenereceptors *ETR2*, *ERS1*, and *ERS2* showed pronounced induction, *ETR1* displayed limited induction, and *EIN4* remained unchanged. Third, the effect of abiotic stress upon expression of ethylene receptors was examined. Microarray analysis indicated that the expression of *ETR1*, *ETR2*, and *ERS1* was reduced after exposure to salt stress, while expression of *ERS2* and *EIN4* remained unchanged. Changes in gene expression were reflected at the protein level as levels of the ETR1

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protein decreased following salt and osmotic stress. A role for ethylene signal transduction in the plants' response to abiotic stress was confirmed by examining the effect of salt and osmotic stress upon the germination of ethylene pathway mutants. My results demonstrate that changes in expression level of ethylene receptors provide a means by which ethylene signal transduction can be activated or repressed.

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CHAPTER I

GENERAL INTRODUCTION

Plants use complex mechanisms to adapt to their environment in order to grow and develop. This is necessary because not only do plants not have the ability to escape from adverse environmental conditions, but also plants have a complicated life cycle, that includes germination, growth, reproduction and senescence. One of the important regulatory mechanisms of adapting to environment is through various signal transduction pathways. Understanding how plants use signal transduction to respond to environmental changes and adjust their growth and development accordingly is interesting and challenging. Until ten years ago, the plant signal transduction pathways that transmit well-known stimuli were viewed as black boxes. However, with advances in modern genetics and plant molecular biology, the black boxes became greyer. Multiple signaling pathways have been recognized in plants that control important processes, such as hormone and light perception, pathogen defense, stress survival, cell division, and circadian rhythms. Although still limited, more and more is being learned about these pathways. This dissertation presents results arising from the study of signal transduction by the plant hormone ethylene. These results focus on the regulation of ethylene receptor levels in *Arabidopsis*.

Arabidopsis As a Model System

One of the approaches that has made it easier for modern plant biologists to elucidate the mechanisms of plant signal transduction is the employment of *Arabidopsis*

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thaliana as a model plant (Patrusky, 1991). *Arabidopsis thaliana* is a modest little flowering weed, commonly called mouse-eared cress. It belongs to the mustard family and is related to a number of vegetables such as broccoli, cabbage, Brussel sprouts, and cauliflower. The features of *Arabidopsis* that attract researchers are its small size, compact genome, predilection to self-pollinate, short life cycle and copious production of tiny seeds. A mature *Arabidopsis* plant is about 30 - 40 cm in height, which is perfect for growing in a laboratory. Its genome is 125 Mb, small in comparison to the genomes of maize and wheat, which are 2,500 Mb and 16,000 Mb, respectively (The *Arabidopsis* Genome Initiative, 2000). Therefore, by using *Arabidopsis*, it is easier to screen for mutants and more convenient for gene cloning. In addition, *Arabidopsis* can be readily transformed with exogenous DNA. Just as yeast is used as a model system to study eukaryotes and *C.elegans* and *Drosophila* as models to study animals, *Arabidopsis* is the most powerful genetic and genomic model system in plant biology.

Actually, *Arabidopsis* has been used for genetic studies for over a century. However, the widespread adoption of *Arabidopsis* as a model plant did not occur until the 1980s. The earliest scientific paper about *Arabidopsis*, describing a mutant plant found in the wild, was published in 1873 (Meyerowitz, 2001). The earliest *Arabidopsis* research was associated with the name of Friedrich Laibach (Meyerowitz, 2001), who used *Arabidopsis* in chromosome studies. Following these initial reports, there were a few publications on genetic studies with *Arabidopsis*. These indicated that *Arabidopsis* had the potential to be a good genetic model organism. In 1975, the advantages of *Arabidopsis* as a model plant were further recognized. A publication that summarized the advantages of adopting *Arabidopsis* as a model system for studying plant development,

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physiology, and molecular genetics was published in *Science* in 1985 (Meyerowitz and Pruitt, 1985). From the 1980s to now, great progress has been made in plant biology, physiology, and molecular genetics using *Arabidopsis* as a model plant (Meyerowitz, 2001).

Ethylene As a Plant Hormone

There are five major classes of plant hormones: auxins, gibberellins, cytokinins, abscisic acid, and ethylene (Kende and Zeevaart, 1997). They function together to regulate physiological events during the growth and development of a plant. Among the five hormones, ethylene has the simplest structure as a two-carbon gaseous alkene. However, this simple structure does not prevent it from having profound effects upon the life of a plant. The biological role of ethylene on plants was discovered over a century ago. It was realized in the mid-nineteenth century that plants around illuminating gas pipelines were often found to have leaves that senesced earlier than plants elsewhere (Abeles et al., 1992). However, it was not until the turn of the century that Neljubow identified that ethylene was the active component of illuminating gas using an assay that determined the effects of ethylene upon the horizontal growth of etiolated pea seedlings (Neljubow, 1901). Since then, more and more physiological effects of ethylene have been revealed. Besides its well-known function in fruit ripening, ethylene also promotes seed germination, inhibits or promotes cell elongation and cell division, and induces abscission and senescence of leaves and flowers (Abeles et al., 1992). Recent studies show that ethylene is also a mediator of adaptation responses to stress and pathogen infection (Abeles et al., 1992; O'Donnell et al., 1996; Penninckx et al., 1996), and

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therefore helps protect plants from environmental challenges such as flood, wind, cold, wounding, and pathogenic attack.

In *planta*, ethylene is biosynthesized from methionine via S-adenosyl-L-methionine (AdoMet) and the cyclic non-protein amino-acid 1-aminocyclopropane-1-carboxylic acid (ACC) (Adams et al., 1979). The ethylene biosynthetic pathway was determined by Yang and co-workers (Fig. 1) (Yang and Hoffman, 1984; Schaller and Kieber, 2002). All vascular plants analyzed to date synthesize ethylene via this pathway. The two key enzymes in this pathway are ACC synthase, which catalyses the conversion of AdoMet to ACC, and ACC oxidase, which catalyses the conversion of ACC to ethylene. The formation of ACC is generally the rate-limiting step in ethylene biosynthesis. The activity of ACC synthase is highly regulated and closely parallels the level of ethylene biosynthesis (Yang and Hoffman, 1984).

Ethylene biosynthesis is regulated by both internal and external factors. The internal factors are associated with the normal life of a plant. During certain stages of growth such as germination, ripening of fruits, abscission of leaves, and senescence of flowers, ethylene biosynthesis is induced. External factors such as mechanical wounding, environmental stresses, and certain chemicals including auxin, and other regulators, can induce ethylene biosynthesis. Exposing a plant to external ethylene can also regulate ethylene biosynthesis. However, this regulation can be either inductive or inhibitory depending on the type of plant (Hoffman and Yang, 1980; Vendrell and McGlasson, 1971).

Etiolated wildtype *Arabidopsis* seedlings, when exposed to ethylene, display a so-called "triple response" (Guzmán and Ecker, 1990), which refers to an enhanced

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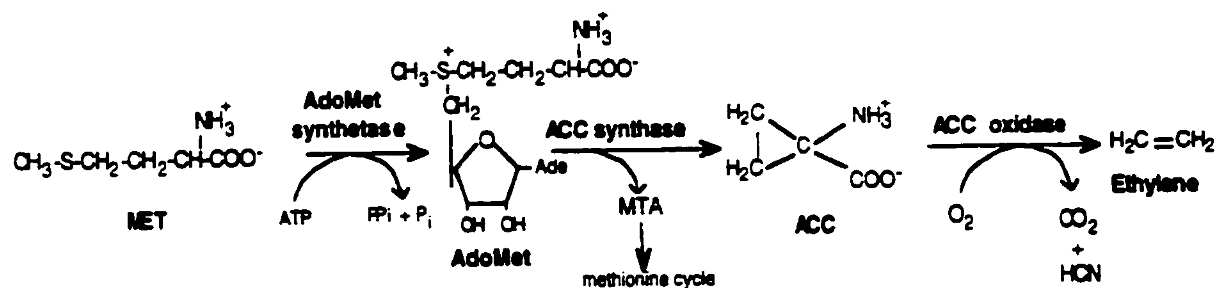

Figure 1. Ethylene biosynthetic pathway (Schaller and Kieber, 2002). The enzymes catalyzing each step are shown above the arrows. AdoMet: S-adenyl-methionine; Met: methionine; ACC: 1-aminocyclopropane-1-carboxylic acid; MTA: methylthioadenine.

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curvature of the apical hook, inhibition of root and hypocotyl elongation, and radial swelling of the hypocotyls and roots. The triple response phenotype has provided a simple means to identify mutants that are defective in ethylene biosynthesis or perception. This screen has allowed the identification of ethylene overproduction mutants, which display a triple response phenotype in the absence of exogenous ethylene and whose phenotype can be reversed by inhibitors of ethylene biosynthesis. It has also allowed for the identification of ethylene insensitive mutants, such as *etr1*, *ein2*, and *ein3*, which fail to display or show a reduced level of triple response in the presence of ethylene. Finally, it has allowed for identification of the Constitutive Triple Response mutant *ctr1*, which shows an irreversible constitutive ethylene response (Guzmán and Ecker, 1990).

The Ethylene Receptor Family of *Arabidopsis* Has Five Members

To date, five genes, *ETR1*, *ETR2*, *ERS1*, *ERS2*, and *EIN4*, have been identified as ethylene receptor genes in *Arabidopsis* (Hua et al., 1995; Hua et al., 1998; Sakai et al., 1998). *ETR1* was the first one of the five genes to be discovered and was subsequently demonstrated to act as an ethylene receptor. In the 1960s, it was already realized that a receptor was needed for the action of ethylene upon plants (Burg and Burg, 1967). However, the ethylene receptor could not be successfully isolated through biochemical methods. In the late twentieth century, the application of molecular genetic studies to *Arabidopsis* resulted in the isolation of ethylene response mutants, including *etr1-1*. The ethylene receptor gene *ETR1* was then isolated by means of map-based cloning (Chang et al., 1993). The facts that demonstrate that ETR1 functions as an ethylene receptor are as follows: (1) *etr1* mutants are dominant and insensitive to ethylene for all ethylene

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responses analyzed (Bleecker et al., 1988); (2) the *ETR1* gene acts upstream of other components of the ethylene pathway according to double-mutant analyses (Kieber et al., 1993); (3) the ETR1 protein is similar to a known family of receptors from bacteria (Chang et al., 1993); and (4) the ETR1 protein binds to ethylene within its amino-terminal domain (Schaller and Bleecker, 1995).

ERS1 was uncovered by cross-hybridization with the *ETR1* gene (Hua et al., 1995). The *ETR2* and *EIN4* genes were identified through mutant screens (Sakai et al., 1998; Hua et al., 1998). *ERS2* was identified by cross-hybridization with the *ETR2* gene (Hua et al., 1998). The deduced proteins of the five members of this gene family share 57 - 79% sequence similarity to each other. The amino-terminal domain is the transmembrane region, which is the most similar region among all the family members. The involvement of *ERS1*, *ERS2*, *ETR2*, and *EIN4* in ethylene sensing is suggested by their sequence similarity to *ETR1* and by their mutant phenotypes. In addition, ERS1 has, like ETR1, been shown to bind to ethylene (Hall et al., 1999).

Loss-of-function mutations in the *ETR1*, *ETR2*, *EIN4* and *ERS2* genes have been isolated (Hua and Meyerowitz, 1998) and the effects of these mutations upon the ethylene response determined. Single loss-of-function mutations in any of these four genes have little or no effect upon the ethylene responses and display a wildtype phenotype. The *etr1;ein4* double loss-of-function mutant has a partial ethylene response-like phenotype in air. The *etr1;etr2;ein4* triple loss-of-function mutant displays a strong ethylene response phenotype in air similar to that of the *ctr1* mutant. The *etr1;etr2;ein4;ers2* quadruple loss-of-function mutant shows a constitutive triple response more severe than that of the *ctr1* mutant. More than half of the quadruple mutants wilted and died before

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bolting and those that survived were infertile. These results indicate that the ethylene receptors are functionally redundant. The fact that knocking out the ethylene receptors does not cause an absence of ethylene response, but rather leads to a constitutive triple response indicates that ethylene receptors are negative regulators of ethylene signaling (Hua and Meyerowitz, 1998).

All five ethylene receptors exhibit sequence similarity to the histidine kinases that function in "two-component" signaling systems. The structural features of the five ethylene receptors are shown in Figure 2. The two-component system is an evolutionarily ancient signaling mechanism, which was first discovered in prokaryotes, and which allows bacteria to respond to various environmental stimuli (Parkinson, 1993; Swanson et al., 1994; Stock et al., 1995). Recently, the yeast osmosensing pathway was also found to function by means of a two-component system (Posas and Saito, 1998). Typically, the two-component system is composed of a sensory histidine kinase and a response regulator. A signal that is initiated by the histidine kinase in response to an environmental stimulus is transmitted through the response regulator via a "His-to-Asp" phospho-relay to its final target, usually a transcription factor.

The histidine kinase domains of ETR1 and ERS1 show good similarity to other histidine kinases. ETR1 and ERS1 also have all the conserved residues considered essential for histidine kinase activity (Chang et al., 1993; Hua et al., 1995), and histidine kinase activity has been demonstrated for ETR1 (Gamble et al., 1998). However, the histidine kinase-like domains of ETR2, ERS2 and EIN4 have significantly diverged. ETR2, ERS2 and EIN4 lack residues considered essential for activity (Hua et al., 1998; Sakai et al., 1998). The resemblance of ethylene receptors to histidine kinases implicates

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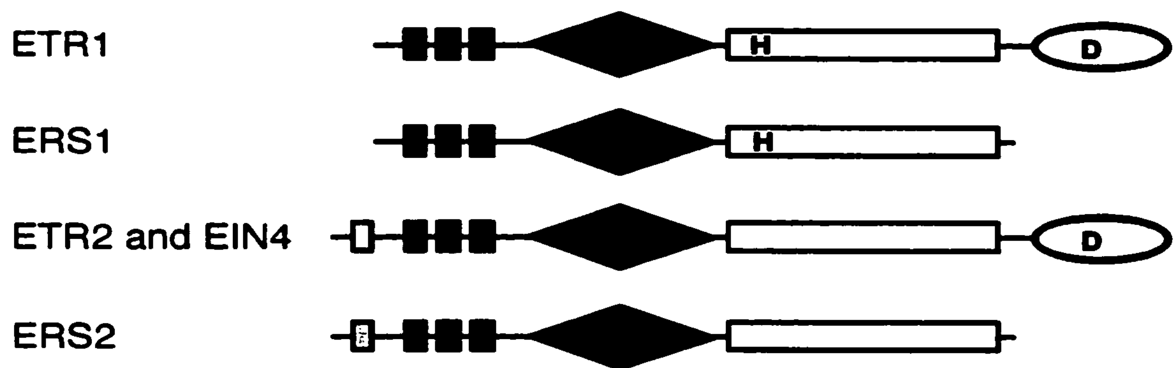

Figure 2. The ethylene receptor family of *Arabidopsis*.
Structural features of the five-member family are indicated. Black bars represent transmembrane domain segments. Gray bars represent putative signal sequences. Diamonds represent GAF domains. Rectangles indicate histidine kinase domains. Ovals indicate receiver domains. The conserved phosphorylation sites upon histidine (H) and aspartate (D) are indicated if present.

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the involvement of a two-component signaling system in ethylene signaling in *Arabidopsis*. However, the function of the histidine kinase in ethylene signaling is still unknown. Recent experiments from the Schaller laboratory suggest that the histidine kinase activity may not play a major role in ethylene signaling (Gamble et al., 2002).

Little is known about the regulation of ethylene receptor expression in *Arabidopsis*. The only information published was based upon northern blot analysis, and indicates that the expression of *ETR1* and *EIN4* gene is not regulated by ethylene, whereas the expression of *ERS1*, *ETR2* and *ERS2* is up-regulated by ethylene (Hua et al., 1998).

Additional Components of The Ethylene Signaling Pathway

In addition to ethylene receptors, other genes involved in ethylene signal transduction in *Arabidopsis* have been cloned and identified as well. The order of action of these genes in ethylene signaling was determined by phenotypic and epistasis (double mutant) analyses (Fig. 3) (Bleecker and Kende, 2000). *CTR1* encodes a Raf-like serine/threonine protein kinase (Kieber et al., 1993). Raf is an element of a mitogen-activated protein kinase (MAPK) signaling cascade. The homology of CTR1 to known MAPKKKs implies that ethylene signaling may operate through a MAP kinase cascade. The product of the *CTR1* gene is a negative regulator of the ethylene response as loss-of-function mutations in *CTR1* confer a constitutive-triple-response phenotype in *Arabidopsis*. Double mutant analysis indicates that *CTR1* acts downstream of the ethylene receptor genes.

EIN2 was demonstrated to act genetically between *CTR1* and *EIN3* (Chao et al., 1997). The product of the *EIN2* gene shows 21% sequence similarity to the eukaryotic

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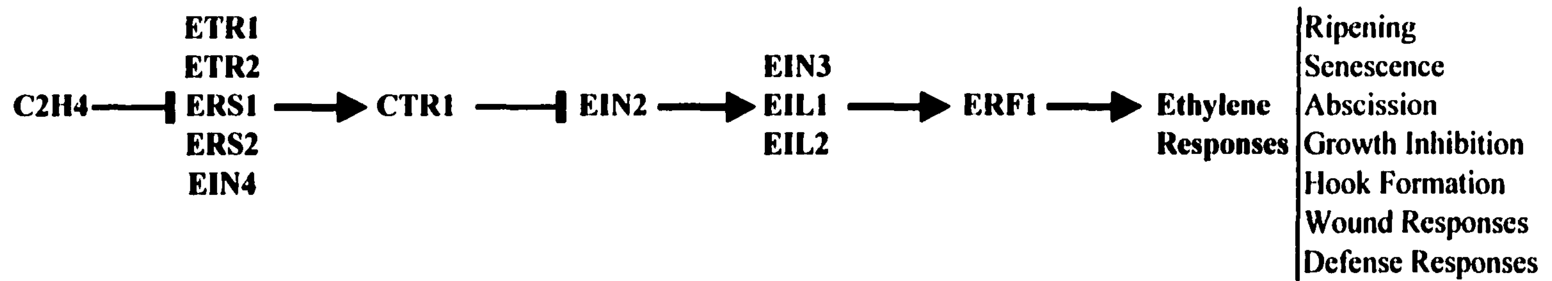

Figure 3. The components of the ethylene signal transduction pathway.
Genes involved in the primary signal transduction pathway are shown.
The ordering of components is based on phenotyic and double mutant analysis.

Nramp family of 12-transmembrane, metal-ion-transporters (Alonso et al., 1999). Phenotypic and epistasis analysis indicate that *EIN2* occupies a central position in the ethylene signaling pathway. The *EIN2* gene has been shown to be involved in multiple hormone responses, stress responses and pathogen responses. However, the function of *EIN2* in the ethylene signaling pathway is still not clear.

The *EIN3* gene encodes a nuclear-localized protein (Chao et al., 1997). Loss-of-function mutations in *EIN3* result in an ethylene insensitive phenotype, suggesting that *EIN3* is a positive regulator in the ethylene pathway (Guzmán and Ecker, 1990). EIN3 and two EIN3-Like (EIL) proteins bind to a promoter element in the *Ethylene-Response-Factor 1* (*ERF1*) gene, indicating that EIN3 and EILs are transcription factors (Solano et al., 1998). Identification of EIN3/EILs as such factors extends known elements of the ethylene response pathway into the nucleus.

The Hypothesis and Approaches of this Dissertation

A hormonal signal transduction can be regulated by varying the concentration of hormone, receptor, or other downstream components. A substantial amount of research has been performed to study the modulation of ethylene biosynthesis in plants. Ethylene biosynthesis can be regulated by environmental stresses, other plant hormones, and by ethylene itself. Regulation of ethylene receptor levels, however, has not been extensively studied. It was only shown recently that expression of the ethylene receptor genes *ERS1*, *ERS2*, and *ETR2* is stimulated by ethylene at the transcriptional level, whereas the expression of *ETR1* and *EIN4* is not appreciably affected by ethylene (Hua et al., 1998). The hypothesis of this dissertation is that ethylene receptor levels are regulated in plants; and that this represents a potential mechanism to control ethylene responses.

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Three different approaches were taken to address this hypothesis. Approaches one and two more clearly defined the effects of ethylene signal transduction upon receptor levels. Approach three identified salt and osmotic stresses as abiotic factors that affect receptor expression, a previously unrecognized effect of these abiotic stresses.

(1) Various mutations in ethylene signaling elements were used to study the intrinsic regulation of the ethylene receptor ETR1 in *Arabidopsis*. Mutations in the ethylene receptor genes and other genes in the pathway have been isolated. To determine whether these mutations affect the expression of ethylene receptors or of other components in the pathway, ETR1 protein levels were quantified in mutant backgrounds containing receptor loss-of-function mutations, ethylene insensitive mutations and constitutive ethylene response mutations. Treatment of wildtype seedlings with silver, which leads to ethylene insensitivity, was also employed to study the effect of ethylene insensitivity upon expression of ethylene receptors. ETR1 protein level was investigated in membrane extracts of these mutant plants by immunoblot analysis. This approach allows the study of how mutations at different steps of the ethylene pathway regulate the expression level of a particular ethylene receptor. This study is described in Chapter II.

(2) Microarray analysis was utilized to investigate genes whose expression level is regulated by ethylene. Instead of the conventional method of treatment with ethylene, a mutant-based approach was employed. Due to the endogenous ethylene biosynthesis in plants, one cannot distinguish the responses originating from endogenous ethylene to that from the exogenous ethylene treatment. This background ethylene effect can be avoided by using reciprocal ethylene pathway mutants *etr1-1* and *ctr1-2*. *etr1-1* is an ethylene insensitive mutant, whereas *ctr1-2* is a constitutive ethylene response mutant. By

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comparing the data generated from *ctr1-2* to that of *etr1-1*, one can mimic the effect of a long-term ethylene treatment on *Arabidopsis*. Genes that were either up-regulated or down-regulated by ethylene were analyzed and categorized. The regulation of ethylene receptors by ethylene was also revealed through this analysis. This study is presented in Chapter III.

(3) The effect of environmental stress upon expression of the ethylene receptor genes was also studied. Salt and osmotic treatment was used as abiotic stress to treat wildtype *Arabidopsis*, and the effect upon expression of the ethylene receptors was investigated by microarray analysis. Changes in gene expression were confirmed at the protein level by immunoblot analysis using an antibody directed against ETR1. To determine if this effect was due to ionic toxicity or osmotic stress, various salts and osmoticums were also tested. The role of ethylene signal transduction in the salt and osmotic stress response was further characterized through examination of ethylene-pathway mutants in response to salt and osmotic stress. This study is described in Chapter IV.

Results from approach one demonstrated that expression of the ethylene receptor gene *ETR1* is affected by mutations within its own coding sequence at the post-transcriptional level, and these post-transcriptional changes contribute to the phenotypes observed in these mutants. The ETR1 protein level did not functionally compensate for the loss of other receptor members. Results from approach two indicated that the effect of ethylene on gene expression exhibits more cases of induction than repression. Forty-five percent of ethylene-induced genes were involved in signaling. In addition to *ETR2*, *ERS1*, and *ERS2*, which had been previously demonstrated ethylene-inducible, *ETR1* is

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also induced by ethylene but to a limited extent. Expression of *EIN4* was not affected by ethylene. Results from approach three demonstrated that the expression of *ETR2*, *ETR1*, and *ERS1* was down-regulated by salt stress at the transcription level, whereas the expression of *ERS2* and *EIN4* remained unchanged. The reduction in transcript level of *ETR1* was also reflected at the protein level in response to salt and osmotic stress. Reduction in the ethylene receptor levels could serve as an alternative mechanism for activating ethylene responses without the presence of ethylene.

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CHAPTER II

EFFECT OF ETHYLENE-PATHWAY MUTATIONS UPON EXPRESSION OF THE ETHYLENE RECEPTOR ETR1

(This chapter is based on a manuscript submitted for publication: Zhao, X-C., Qu, X., Mathews, D.E., Schaller, G.E. Effect of Ethylene Pathway Mutations upon Expression of the Ethylene Receptor ETR1 from *Arabidopsis*.)

Introduction

Ethylene (C_2H_4) is a simple gaseous hydrocarbon that has profound effects upon plant growth and development. Ethylene regulates seed germination, seedling growth, leaf and petal abscission, organ senescence, ripening, stress responses, and pathogen responses (Mattoo and Suttle, 1991; Abeles et al., 1992). An important contribution to our understanding of ethylene signal transduction has come from the generation of mutants in *Arabidopsis* with altered ethylene sensitivity (Chang and Shockey, 1999; Stepanova and Ecker, 2000). These mutations fall into two main classes: (1) mutations that render a plant insensitive to ethylene and (2) mutations that result in a constitutive ethylene response. Characterization of *Arabidopsis* mutants has led to the identification of ethylene receptors and additional components in the ethylene signal transduction pathway.

The ethylene receptor family of *Arabidopsis* contains five members (ETR1, ETR2, ERS1, ERS2, and EIN4) (Schaller, 2000; Chang and Stadler, 2001), with ethylene

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binding confirmed for ETR1 and ERS1 (Schaller and Bleecker, 1995; Rodriguez et al., 1999; Hall et al., 2000). The receptors contain three amino-terminal transmembrane domains that encompass the ethylene-binding site (Schaller and Bleecker, 1995; Rodriguez et al., 1999). The binding site contains a copper cofactor that is required for the high-affinity ethylene binding that receptors display (Rodriguez et al., 1999). In the carboxyl-terminal half, the receptors contain regions with homology to histidine kinases and in some cases the receiver domains of response regulators (Schaller, 2000; Chang and Stadler, 2001), signaling elements originally identified as parts of bacterial two-component systems (Parkinson, 1993; Schaller, 2000). Histidine kinase activity has been confirmed *in vitro* for ETR1 (Gamble et al., 1998), but the role of this activity in signal output is still unclear (Gamble et al., 2002).

Mutations in the ethylene receptors can result in ethylene insensitivity or constitutive ethylene responses, depending on the nature of the mutation. Ethylene-insensitivity can result from single amino-acid changes within the region of the receptor involved in ethylene binding (Chang et al., 1993; Hua et al., 1995; Hua et al., 1998; Sakai et al., 1998). Evidence indicated that these gain-of-function mutations either disrupt ethylene binding or uncouple ethylene binding from signal output (Schaller and Bleecker, 1995; Hall et al., 1999; Rodriguez et al., 1999). For example, the *etr1-1* mutation abolishes the ability of the receptor to coordinate the copper cofactor and as a consequence eliminates ethylene binding (Rodriguez et al., 1999). The ethylene-insensitive mutations are dominant and a single mutation in any one of the five family members can confer ethylene insensitivity. Loss-of-function mutations have been identified in four out of five members of the ethylene receptor family (Hua and

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Meyerowitz, 1998). Single loss-of-function mutations have little or no effect upon ethylene signal transduction. However, in combination with the *ETR1* loss-of-function mutation, the mutants show constitutive ethylene responses and this effect is most pronounced in triple and quadruple loss-of-function mutations (Hua and Meyerowitz, 1998). These results indicate that there is functional overlap among the receptor family members. These results also indicate that the receptors serve as negative regulators of the ethylene response pathway since elimination of receptors activates ethylene responses.

Additional elements involved in ethylene signal transduction have also been identified by mutational analysis in *Arabidopsis*. RAN1 is a copper-transporting ATPase apparently required for addition of the copper cofactor to the ethylene receptors (Hirayama et al., 1999; Woeste and Kieber, 2000). Mutations in *RAN1* alter ethylene signal transduction, a loss-of-function mutation in resulting in a constitutive ethylene response. CTR1, EIN2, and EIN3 are all thought to act in the same primary response pathway as the ethylene receptors. CTR1 belongs to the Raf family of protein serine/threonine kinases that initiate MAP kinase cascades in eukaryotes and, based on genetic evidence, acts downstream of the ethylene receptors (Kieber et al., 1993). CTR1 has been shown capable of physical interaction with the ethylene receptors ETR1 and ERS1. Loss-of-function mutations in *CTR1* result in constitutive ethylene responses. EIN2 is an integral membrane protein with similarity to the Nramp family of metal-ion transporters and, based on genetic evidence, acts downstream of CTR1 (Alonso et al., 1999). Loss-of-function mutations in *EIN2* result in ethylene insensitivity. EIN3 belongs to a family of transcription factors that are directly activated by the ethylene signal transduction system and are required for ethylene-dependent gene induction (Chao et al.,

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1997). EIN3 functions downstream of EIN2, and loss-of-function mutations in *EIN3* render a plant ethylene-insensitive.

The effect of ethylene pathway mutations upon expression of the ethylene receptor ETR1 was analyzed. This analysis was facilitated by the following: (1) the availability of a number of mutations within the receptor itself, thereby providing independent verification for the effects of these mutations; (2) the availability of an antibody against ETR1, thereby allowing for analysis at the protein level; and (3) a detectable basal level of expression for ETR1, thereby allowing increases and decreases in expression to be determined. My results lend insight into how ethylene receptor mutations affect expression of the ethylene receptor ETR1, and indicate that mutations within ETR1 can result in post-transcriptional changes in expression level. My results also lend insight into the mechanism by which mutations within the receptors can lead to dominant ethylene insensitivity.

Materials and Methods

Plant Material and Growth Conditions

Arabidopsis thaliana mutants in the ecotype Columbia were used for all experiments. Seedlings were grown on 1.0% (w/v) agar plates of half-strength Murashige and Skoog basal medium (pH 5.65) with Gamborg's vitamins (MS media, Sigma) in the dark. Seeds were surface-sterilized with 70% ethanol and stratified for 2 days at 4°C prior to growth at 22°C. Seeds were exposed to light for 12 hours, then incubated in the dark. Seedlings were typically examined after 4 d, time 0 corresponding to when the plates were removed from 4°C and brought to 22°C. To measure hypocotyl length,

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seedlings were grown on vertically oriented plates. Seedlings on the plates were scanned using Adobe Photoshop (Version 5.5) and a LaCie scanner, and measured using NIH Image (Version 1.60, National Institute of Health, Bethesda, MD). For analysis of the *ran1-3* mutant, seedlings from a segregating population were grown for 4 weeks under an 8-hour light cycle to allow for maximal rosette development prior to harvest. Homozygous *ran1-3* seedlings were identified based on their readily distinguishable constitutive-ethylene-response phenotype (Woeste and Kieber, 2000).

Protein Isolation and Immunoblot Analysis

For isolation of *Arabidopsis* membranes, plant material was homogenized at 4 °C in extraction buffer (50 mM Tris, pH 8.5, 150 mM NaCl, 10 mM EDTA, 20% [v/v] glycerol) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL pepstatin, 10 μg/mL aprotinin, and 10 μg/mL leupeptin as protease inhibitors. The homogenate was strained through Miracloth (Calbiochem-Novobiochem, San Diego) and centrifuged at 8,000*g* for 15 min. The supernatant was centrifuged at 100,000*g* for 30 min, and the membrane pellet was resuspended in 10 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 10% (v/v) glycerol with protease inhibitors. Protein concentration was determined by a modification of the Lowry assay (Lowry et al., 1951) in which samples were treated with 0.4% sodium deoxycholate (Schaller and DeWitt, 1995). Bovine serum albumin was used as a standard for protein assays.

For immunoblot analysis, membranes were mixed with SDS-PAGE loading buffer and incubated at 37 °C for 1 hr. Proteins were fractionated by SDS-PAGE using 8% (w/v) polyacrylamide gels (Laemmli, 1970). After electrophoresis, proteins were either stained with Coomassie blue or electrotransferred to Immobilon nylon membrane

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(Millipore). Western blotting was performed by using anti-ETR1(401-738), or anti-H^+-ATPase polyclonal antibodies. Anti-ETR1(401-738) was prepared against a GST-fusion protein with amino acids 401-738 of ETR1 (Schaller et al., 1995). The anti-H^+-ATPase antibody (DeWitt et al., 1996) was provided by Dr. M. Sussman (University of Wisconsin, Madison). Immunodecorated proteins were visualized by enhanced chemiluminescence detection according to the manufactuer (Pierce). Densitometric analysis was performed by using NIH Image after first scanning the exposed film and then capturing the images with Adobe Photoshop. The relative expression level for ETR1 was quantified by comparison to a dilution series of ETR1.

Northern Blot Analysis (This part of the work was performed by Xiang Qu.)

Total RNA was extracted from etiolated *Arabidopsis* seedlings according to the method of Carpenter and Simon (Carpenter and Simon, 1998). mRNA was isolated from total RNA using the PolyATract mRNA isolation system (Promega). For northern blot analysis, RNA was separated on 1% agarose gels containing glyoxal and dimethylsulfoxide using the NorthernMax-Gly kit (Ambion) according to the manufacturer's instructions. RNA was transferred to a nylon membrane by the capillary method and fixed by UV cross-linking. Hybrizations were performed using buffers supplied with the NorthernMax-Gly kit. Single-stranded DNA anti-sense probes were made using primers designed to anneal at the 3' end of the coding sequences of the selected genes. Radiolabeled probes were made and the blot stripped between hybridizations by using the Strip-EZ PCR kit (Ambion) according to the manufacurer's instructions. Radioactivity was imaged and quantitated by phosphorimaging with a Molecular Imager FX (BioRad), using Quantity One software.

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Results

Effect of Ethylene-Insensitivity Conferring Mutations upon Expression of ETR1

Four dominant mutations have been isolated in *ETR1* that confer ethylene insensitivity on plants. These mutations, designated *etr1-1*, *etr1-2*, *etr1-3*, and *etr1-4*, all result in single amino acid changes within the hydrophobic domain of ETR1 that has been implicated in ethylene binding (Fig. 4A) (Chang et al., 1993). The *etr1-1*, *etr1-3*, and *etr1-4* mutations either reduce or eliminate ethylene binding (Hall et al., 1999). The *etr1-2* mutation does not disrupt ethylene binding, but apparently uncouples ethylene binding from signal output (Hall et al., 1999). Based on immunoblot analysis, the protein levels of the mutant receptors etr1-1, etr1-2, etr1-3, and etr1-4 were all approximately 2 to 3 fold higher than that of the wildtype receptor ETR1 when analyzed in etiolated seedlings (Fig. 4B). To determine if the effect upon expression occurred at the transcriptional or post-transcriptional level, transcript levels of the receptor were determined by northern blot analysis in both wildtype and *etr1-1* backgrounds (Fig. 4C). No difference in transcript levels was found for the receptor in the mutant background. However, it was found that the etr1-1 protein was present at 2-fold higher levels than the ETR1 protein when analyzed by immunoblot using a portion of the same plant material examined by northern blot (Fig. 4C). Thus the increase in expression of ethylene-insensitive mutations of ETR1 occurs at the post-transcriptional level.

To determine if increased expression of the receptor was restricted to mutant lesions in ETR1 or was a general feature of ethylene insensitivity in *Arabidopsis*, I examined other ethylene-insensitive mutations. Seedlings were examined that contained dominant ethylene-insensitive mutations in other ethylene receptors (*etr2-1* and *ein4-1*).

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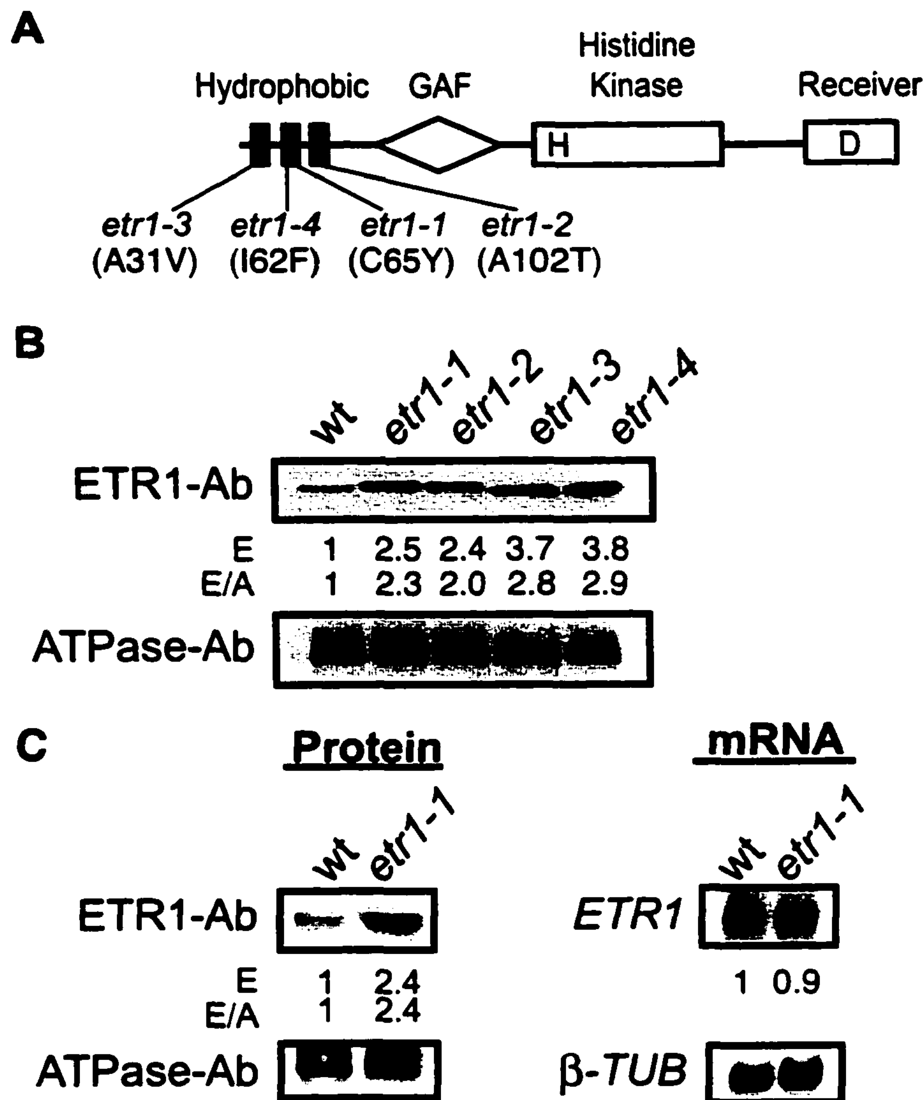

Figure 4. Effect of ethylene-insensitive mutations upon expression of ETR1.
A, Structure of ETR1 and position of ethylene insensitive mutations. The hydrophobic ethylene-sensing domain, the GAF domain, the histidine kinase domain, and the receiver domain are indicated. H and D indicate putative phosphorylation sites. B, Immunoblot analysis of wildtype and ethylene-insensitive mutants of ETR1. Etiolated seedlings were grown for 4 days, and the level of immunodetectable full-length receptor then determined from 10 μg membrane protein using an antibody directed against ETR1. Expression levels were quantified densitometrically (E) and also normalized against immunologically determined levels of the H^+-ATPase (E/A) as an internal control. C, Northern blot analysis of mRNA obtained from wildtype and *etr1-1* seedlings (Performed by Xiang Qu) and immuno blot analysis of the corresponding protein level. Blots were probed with an *ETR1* probe and a β-tubulin gene probe as an internal control. The numbers represent the expression level of the ethylene receptor gene after normalization for the level of β-tubulin expression. Protein level is also shown by immunoblot analysis of the same plant material used for Northern blot analysis.

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Seedlings were also examined that contained ethylene insensitive mutations in the downstream ethylene signaling components *EIN2* and *EIN3*. The expression level of ETR1 based on immunoblot in these other mutant backgrounds was comparable to or less than that found in the wildtype background (Fig. 5A, B). Thus the increased expression of ethylene-insensitive mutants of ETR1 is restricted to those lesions present in ETR1 itself, rather than being a general feature of ethylene-insensitive mutations.

Some chemical compounds are able to induce ethylene insensitivity in plants by interacting with the ethylene receptors. Silver is thought to replace the copper cofactor present in the ethylene-binding site of the receptor. Receptors containing silver are still able to bind ethylene but the binding site is apparently perturbed such that ethylene binding is uncoupled from signal output (Rodriguez et al., 1999). Here I hypothesized that binding of silver by an ethylene receptor might mimic the effect of an ethylene-insensitive mutation in that receptor and result in an increased expression level of the receptor. Consistent with this hypothesis, it was observed that wildtype seedlings treated with 10 μg/mL silver nitrate had higher levels of ETR1 than control untreated seedlings based upon immunoblot analysis (Fig. 6A). The stimulatory effect of silver upon expression was lacking with ethylene-insensitive mutations of ETR1 (Fig. 6A), consistent with binding of silver to the ethylene receptor ETR1 affecting protein levels in a manner analogous to the ethylene-insensitive mutants of ETR1. Seedlings that contain mutations in the downstream ethylene signaling components *CTR1*, *EIN2* and *EIN3* were also treated with silver nitrate. The expression level of ETR1 based on immunoblot in treated seedlings was also higher than in control untreated seedlings (Fig.6B). Thus the effect of

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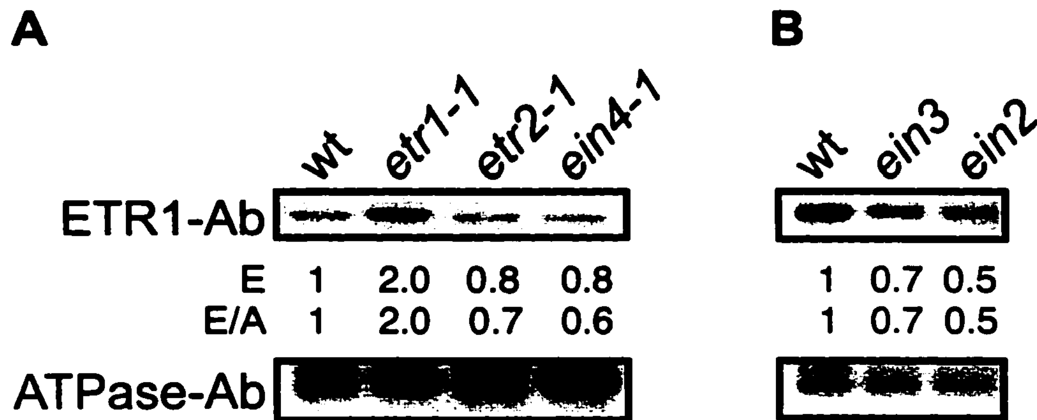

Figure 5. Effect of other ethylene-insensitive mutants upon expression of ETR1. A, Immunoblot analysis of ETR1 levels in wildtype and ethylene-insensitive mutants of *ETR2* and *EIN4*. B, Immunoblot analysis of wildtype and downstream ethylene-insensitive mutants *ein3* and *ein2*. Etiolated seedlings were grown for 4 days, and the level of immunodetectable full-length receptor then determined from 10 μg membrane protein using an antibody directed against ETR1. Expression levels were quantified densitometrically (E) and also normalized against immunologically determined levels of the H^+-ATPase (E/A) as an internal control.

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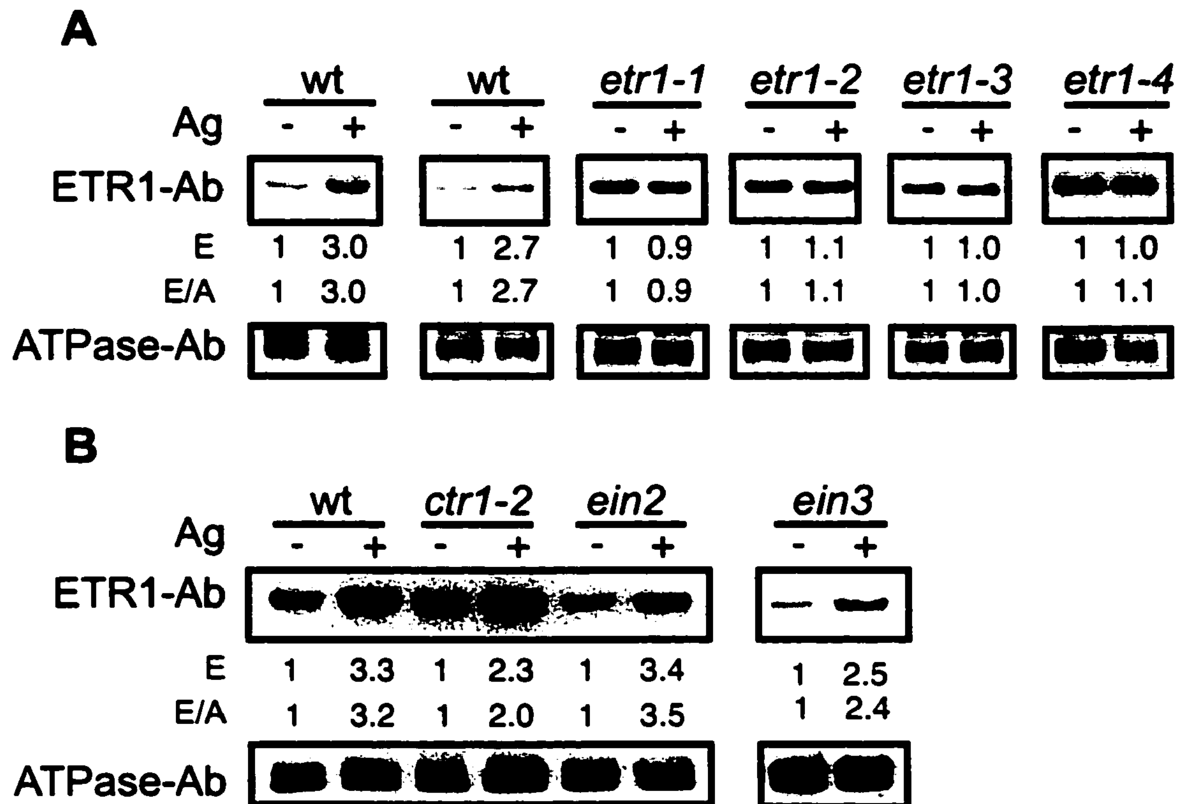

Figure 6. Effect of silver treatment upon expression of ETR1.
Etiolated seedlings of wildtype and *etr1* mutant (A), and wildtype and *ctr1-2, ein2* and *ein3* mutant (B) were grown in the presence or absence of 10 μg/mL silver nitrate (Ag). Immunoblot analysis was then performed using antibodies directed against ETR1 and the H^+-ATPase as an internal control on 10 μg membrane protein. Expression levels are given based directly upon that determined with anti-ETR1 antibody (E) and normalized against the ATPase levels (E/A). For each plant background, expression level of the receptor in the presence of silver is given relative to that observed in the absence of silver. Results from three independent experimental treatments of wildtype plants with silver are shown.

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silver upon the protein level of ETR1 is independent of the action of the downstream ethylene signaling components, and that is also analogous to the effect of *etr1* mutations.

Effect of Loss-of-Function Mutations in other Ethylene Receptors upon Expression of ETR1

Loss of one member in a gene family can sometimes lead to functional compensation, whereby expression of another member of the same gene family is induced to compensate for activity of the missing family member (Bérard et al., 1997; Mulligan et al., 1998; Minkoff et al., 1999). An intriguing set of experiments suggests that functional compensation occurs within the ethylene receptor family of tomato (Tieman et al., 2000). Antisense was used to reduce expression of the gene encoding the tomato ethylene receptor NR. These transgenic tomato lines exhibited normal ethylene responses but had increased mRNA levels for the ethylene receptor LeETR4, indicating that *LeETR4* functionally compensates for a reduction in *NR* gene expression.

The *Arabidopsis* ethylene receptor ETR1was examined to determine if its expression was affected by loss-of-function mutations in other ethylene-receptor family members. Analysis was performed on single loss-of-function mutants (*etr2-3* and *ein4-4*), a double mutant (*etr2-3*; *ein4-4*), and a triple mutant (*etr2-3*; *ein4-4*; *ers2-3*) (Hua and Meyerowitz, 1998). The single mutants have little effect upon growth of etiolated *Arabidopsis* seedlings, but seedlings containing the double and triple mutants demonstrate partial induction of the triple-response phenotype, consistent with loss of receptors activating the ethylene response pathway (Fig. 7A) (Hua and Meyerowitz,

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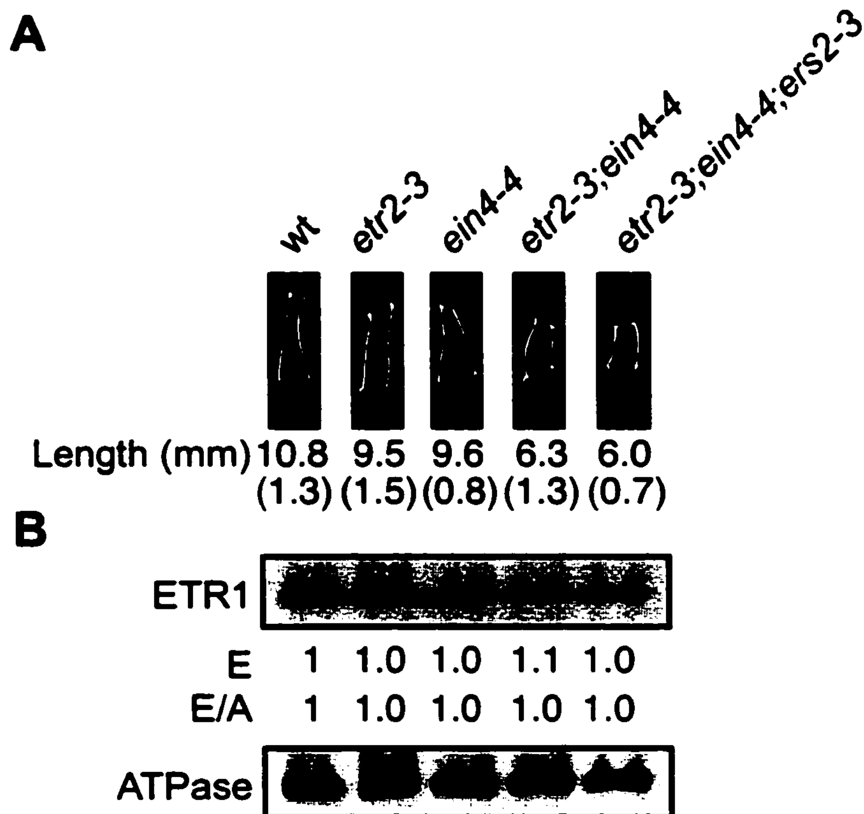

Figure 7. Effect of loss-of-function mutations in *ETR2*, *EIN4*, and *ERS2* upon expression of ETR1. The phenotypes of 4-day-old dark-grown seedlings containing single, double, and triple mutant combinations of *etr2-3*, *ein4-4*, and *ers2-3* are shown. The mean hypocotyl length is given in mm based on measurement of at least 25 seedlings with the standard deviation in parentheses. Immunoblot analysis was performed using antibodies directed against ETR1 and the H^+-ATPase as an internal control on 10 μg membrane protein. Expression levels are given based directly upon that determined with anti-ETR1 antibody (E) and normalized against the ATPase levels (E/A).

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1998). The expression level of ETR1 protein in these mutant backgrounds was comparable to that found in the wildtype background (Fig. 7B), indicating that ETR1 did not functionally compensate for the loss of these other members of the receptor family.

Effect of mutations in *RAN1* and *CTR1* upon expression of ETR1

RAN1 is a copper-transporting ATPase implicated in the delivery of the copper cofactor to the ethylene receptors (Hirayama et al., 1999; Woeste and Kieber, 2000). The *ran1-1* and *ran1-2* mutations cause single amino acid changes in the RAN1 protein and are thought to alter rather than eliminate function (Hirayama et al., 1999). Plants containing these mutations demonstrate an induction of ethylene responses when treated with *trans*-cyclooctene, normally an antagonist of ethylene responses, but have no other discernable effect upon growth (Hirayama et al., 1999). Both *ran1-1* and *ran1-2* seedlings expressed ETR1 at levels similar to wild type seedlings (Fig. 8A). The loss-of-function mutation *ran1-3* results in constitutive activation of the ethylene response pathway. Because *ran1-3* also results in lethality after rosette formation (Woeste and Kieber, 2000), homozygous *ran1-3* plants were identified based on phenotype from a segregating population of 4-week-old plants grown in the light. No difference was observed in ETR1 levels in *ran1-3* plants compared to wildtype plants or members of the segregating population that lacked the *ran1-3* phenotype (Fig. 8A). Loss-of-function mutations in the serine/threonine kinase CTR1 also result in constitutive ethylene responses. Typically, a 2-fold increase was observed in levels of ETR1 in the *ctr1-2* mutant background relative to wild type under dark growth conditions (Fig. 8B). This

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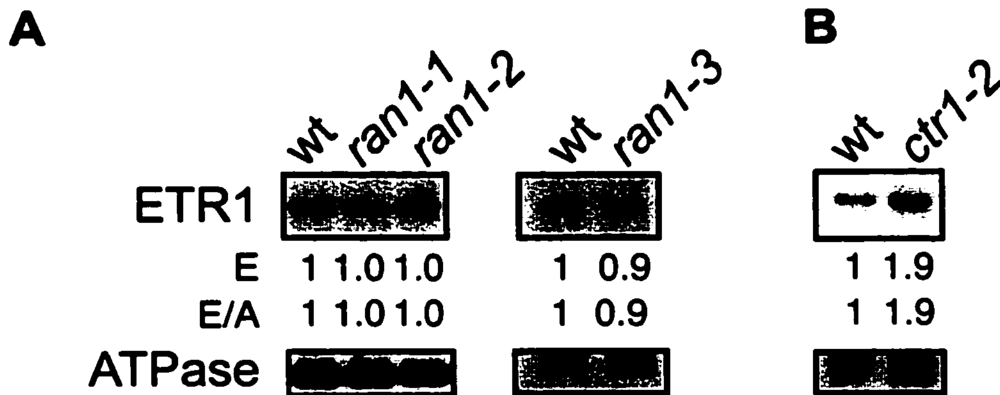

Figure 8. Effect of mutations in *RAN1* and *CTR1* upon expression of ETR1. Immunoblot analysis was performed using antibodies directed against ETR1 and the H^+-ATPase as an internal control. Expression levels are given based directly upon that determined with anti-ETR1 antibody (E) and normalized against the ATPase levels (E/A). A, Effect of ran1 mutations on expression of ETR1. For *ran1-1* and *ran1-2*, etiolated seedlings were examined; for *ran1-3*, leaves of 4-week-old plants were examined. B, Effect of the *ctr1-2* mutation upon expression of ETR1 in etiolated seedlings.

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could arise due to a low level of ethylene inducibility for the *ETR1* transcript (Hua et al., 1998).

Discussion

Genetic analysis supports the model shown in Figure 9 whereby ethylene receptors negatively regulate ethylene responses in the plant (Hua and Meyerowitz, 1998; Bleecker, 1999). According to this model, wildtype ethylene receptors actively repress ethylene responses in the air. In the presence of ethylene, wildtype receptors switch to a signaling-inactive state that allows for induction of ethylene responses (Fig. 9). The etr1-1 mutant receptor of *Arabidopsis* is apparently locked into the signaling state that it has in air, such that it represses ethylene responses even in the presence of ethylene (Fig. 9) (Schaller and Bleecker, 1995). The dominant *etr1-1* mutation arises due to a single amino-acid change (Cys65Tyr) that results in an inability to bind the copper cofactor in the ethylene binding site, and consequently an inability to bind ethylene. Expression analysis of ethylene pathway mutants refines the model on how ethylene insensitivity is conferred by ETR1 mutants.

Each of the four ethylene-insensitive mutations of ETR1 results in increased protein levels of the receptor. This result can be phenocopied at the molecular level by treatment of plants with silver, which is also capable of generating ethylene insensitivity in plants. Both the ethylene-insensitive mutations and silver are likely to perturb the ethylene-binding site. All four ethylene-insensitive mutations in ETR1 lie within the transmembrane domains implicated in ethylene binding, and three of the mutations have been shown to reduce or eliminate ethylene binding (Hall et al., 1999). Silver is thought

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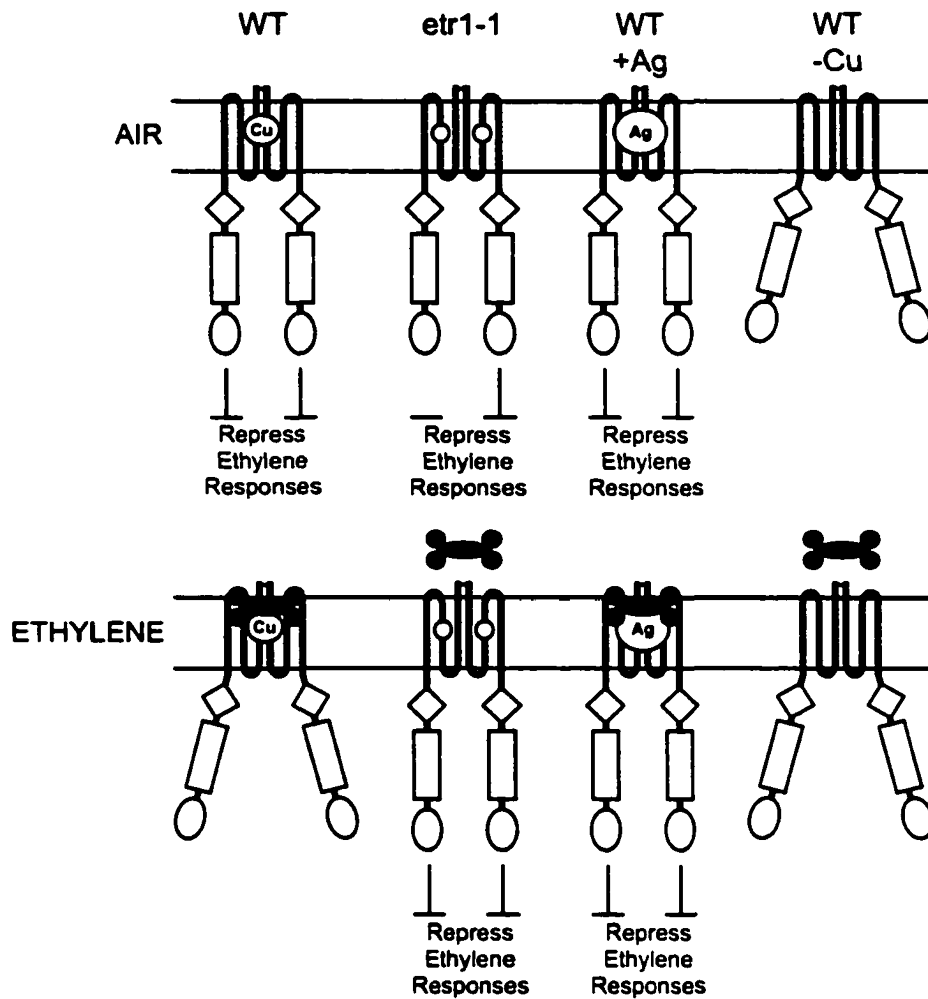

Figure 9. A model for signaling by wildtype and mutant versions of the ethylene receptor ETR1. The ethylene receptor ETR1 contains one ethylene binding site per homo-dimer, with ethylene-binding mediated by a single copper ion (Cu) present in the ethylene binding site. In air, wildtype (WT) receptors actively repress ethylene responses. In ethylene, wildtype receptors are inactivated, thereby relieving repression of the ethylene-response pathway. The etr1-1 mutation (indicated by an open circle) eliminates binding of the copper co-factor and locks the receptor into a conformation such that the receptor represses ethylene responses even in the presence of ethylene. The replacement of the copper co-factor by silver (WT+Ag) also serves to lock the receptor into a conformation such that it continuously represses ethylene responses. In contrast, elimination of the copper co-factor (WT-Cu) results in the receptor adapting an inactive conformation in air and ethylene.

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replace the copper cofactor present in the ethylene binding site of the receptor, generating a receptor still capable of binding ethylene but incapable of transducing the ethylene signal (Fig. 9) (Rodriguez et al., 1999). No difference in transcript levels between *etr1-1* and wild type *ETR1* was observed, thus the differences in expression are post-transcriptional. The ethylene-insensitive forms of the receptor may have a slower rate of turnover than the wild type receptors. Turnover of animal hormone receptors is commonly regulated by hormone binding (Wiley, 1992), with the ligand-bound receptor endocytosed and degraded, and it is possible that turnover of plant ethylene receptors is also regulated through a conformational change brought about by ligand binding. In such case, endogenous ethylene levels within the plant would have to be sufficient to result in differing rates of turnover for the wildtype and mutant receptors.

The discovery that ethylene-insensitive mutants of ETR1 have a higher expression level than wildtype receptors helps resolve a paradox in our understanding of signaling by ethylene receptors. An ethylene-insensitive mutation in one member of the five-member ethylene-receptor family is sufficient to confer ethylene insensitivity, suggesting that signaling by one family member is enough to repress ethylene responses. On the other hand, loss-of-function mutations in three receptors are sufficient to induce ethylene responses (Hua and Meyerowitz, 1998), a situation under which two family members would still theoretically be signaling to repress ethylene responses. It has thus been proposed that mutant receptors such as etr1-1 may be hyperactive (Hua and Meyerowitz, 1998; Bleecker, 1999; Chang and Stadler, 2001). My data indicate that the signal output by an ethylene-insensitive mutant receptor is not equivalent to that of a wild type receptor, due to the difference in expression levels. An increase in expression of the

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ethylene-insensitive mutants of ETR1 can help account for their apparent hyperactivity, as this would result in an increase in signal output and the ability to repress ethylene responses. Other mechanisms may also increase hyperactivity of the ethylene-insensitive mutant receptors, such as their postulated ability to convert wildtype receptors to an ethylene-insensitive signaling state via heteromeric interactions (Chang and Stadler, 2001; Gamble et al., 2002)

Analysis of ETR1 expression in the *ran1-3* background further clarifies the mechanism by which mutations in ethylene receptors confer ethylene insensitivity. The *ran1-3* mutant eliminates a copper transporter required for delivery of the copper cofactor to the ethylene receptors (Hirayama et al., 1999; Woeste and Kieber, 2000). Plants containing the *ran1-3* mutation display a constitutively active ethylene response (Woeste and Kieber, 2000). Interestingly, mutations like *etr1-1* that produce a receptor unable to bind the copper cofactor result in the opposite phenotype: ethylene insensitivity (Rodriguez et al., 1999). This difference in phenotypes could be due to destabilization of the ethylene receptors in the *ran1-3* background or functional differences between receptors lacking copper and ethylene-insensitive receptor mutations. My data support the second hypothesis. ETR1 protein was detected in the *ran1-3* background at similar levels to that found in the wildtype background indicating that, although the receptor is present and lacking the copper cofactor, it does not confer ethylene insensitivity. Presumably, protein levels of the other members of the ethylene receptor family are similarly unaffected. Thus, wildtype ethylene receptors lacking the copper cofactor have a loss-of-function phenotype (i.e. the *ran1-3* mutation produces the same constitutive ethylene-response phenotype found in plant lines containing multiple loss-of-function

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mutations in the ethylene receptors). Wildtype receptors lacking the copper cofactor may adopt a signaling-inactive conformation similar to the conformation of wildtype receptors that have ethylene bound (Fig. 9). The amino-acid changes that result from mutations like *etr1-1* (Cys65Tyr) result in a gain of function, as they prevent not only copper binding but also lock the receptor into a signaling-active conformation such as it has in air (Fig. 9). The proposal that receptors in the *ran1-3* background are not equivalent to receptors containing ethylene-insensitive mutations is consistent with the finding that the ethylene-insensitive *etr1-3* mutant can suppress the *ran1-3* constitutive-ethylene-response phenotype (Woeste and Kieber, 2000). The finding that ETR1 is still present in the *ran1-3* background also raises the possibility that not all mutations that eliminate ethylene binding will as a consequence confer ethylene insensitivity.

Loss-of-function mutations in other members of the ethylene receptor family had little effect upon expression of ETR1, indicating that ETR1 does not functionally compensate for the loss of these receptors. Functional compensation was previously found among members of the ethylene-receptor family from tomato. In tomato, a reduction in expression of the gene encoding the ethylene-receptor NR resulted in increased mRNA levels for the ethylene receptor LeETR4 (Tieman et al., 2000). Nevertheless, no evidence was observed that the expression of *ETR1* increased to functionally compensate for a decrease in the expression of three other members of the receptor family.

In summary, the results described here clarify the mode of action of ethylene pathway mutations previously identified in *Arabidopsis*. Protein level of ETR1 was particularly sensitive to mutations within its own coding sequence, but was affected to

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only a limited extent by mutations in other pathway components. Ethylene-insensitive mutations within the ethylene binding site of *ETR1* affected expression of *ETR1* itself at the post-transcriptional level, and these post-transcriptional changes contribute to the phenotypes observed in these mutants. The receptor appears capable of assuming two different conformations, one active and the other inactive for signal output, and modifications within the ethylene binding site can result in the receptor becoming locked into either of these conformations. In the work described here, the *etr1-1* mutation (Cys65Tyr) and silver binding would lock the receptor into the active conformation, while simple loss of the copper cofactor as mediated by *ran1-3* would lock the receptor into the inactive conformation.

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CHAPTER III

ANALYSIS OF ETHYLENE-REGULATED GENES REVEALED BY USE OF RECIPROCAL MUTANTS IN *ARABIDOPSIS*

Introduction

Among the five classical plant hormones, ethylene has the simplest chemical structure: a two-carbon gaseous alkene. However, its simple structure does not prevent it from having profound effects upon plant growth and development. Ethylene is perceived by a receptor family that consists of five members, ETR1, ERS1, ETR2, ERS2, and EIN4 (Schaller, 2000; Chang and Stadler, 2001). These receptors have been identified as negative regulators of ethylene responses based on mutational studies (Hua and Meyerowitz, 1998). *CTR1*, *EIN2* and *EIN3* are downstream elements involved in ethylene signal transduction that have been identified through mutant analysis and phenotypic studies (Kieber et al., 1993; Chao et al., 1997: Alonso et al., 1999). The *EIN3* gene encodes a nuclear-localized protein (Chao et al., 1997). EIN3 and two EIN3-like (EIL) proteins are transcription factors that have been demonstrated to bind to a promoter element in the *Ethylene-Response-Factor1* (*ERF1*) gene (Solano et al., 1998). The ERF1 is also a transcription factor, which binds to the GCC box and activates the expression of secondary ethylene response genes such as *basic-chitinase* and defensin (*PDF1.2*) (Solano et al., 1998). Thus the transcriptional regulation in ethylene signal transduction is not only at the primary level, but at the secondary level as well (Solano et al., 1998).

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The study of ethylene-regulated gene expression is a key to understanding how this multi-level transcriptional cascade transduces signals to its final targets.

To identify genes that are up- or down-regulated in response to ethylene, DNA microarray technology was employed (Schena et al., 1995). In addition to drastic changes in mRNA levels that can be identified by differential display and other techniques, the high sensitivity of DNA microarray technology allows the detection of more subtle changes in gene expression (Kehoe et al., 1999). In addition to identification of genes with an increase or a decrease in transcript levels, microarray analysis can also identify genes that are unaffected or are very low in abundance. Therefore an integrated analysis of gene expression patterns can be obtained. Such broad-based views of genome-wide gene expression patterns provide a powerful tool for elucidation of the gene regulatory network (Schaffer et al., 2001).

The conventional way to examine the effects of hormones on plant gene expression by microarray is to treat the plant with a hormone and compare the response to an untreated control. The advantage of this method is that it allows the detection of transitory and long-term effects by simply varying the length of treatment. However, the limitation of this approach is that it cannot distinguish the effects caused by the endogenous hormone production from the effects caused by the hormone treatment. Previous studies regarding ethylene-responsive genes in plants were performed by means of ethylene treatment (Hua et al., 1998; Schenk et al., 2000). To eliminate the background due to endogenous ethylene biosynthesis, reciprocal mutants in the ethylene signaling pathway were used in this study. *etr1-1* is an ethylene-insensitive mutant arising from a single amino acid change in the ethylene-binding domain of *ETR1*

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(Bleecker et al., 1988). *ctr1-2* is a constitutive ethylene response mutant due to a loss-of-function mutation in *CTR1* (Kieber et al., 1993). By comparing the microarray data generated from the *ctr1-2* plants to that from the *etr1-1* plants, one can mimic a maximal ethylene effect on gene expression without the interference of endogenous ethylene. Thus genes that are induced or repressed by ethylene can be identified by simply comparing two sets of microarray data.

My results demonstrate that ethylene regulates the expression of genes involved in many physiological aspects of the plant. Genes that are involved in signal transduction were the main target of regulation. In particular, a number of genes that are involved in ethylene biosynthesis and ethylene signaling are induced by ethylene. My results also indicate that the use of reciprocal mutants with microarray analysis provides a simple and effective approach to accurately study gene expression in plants.

Materials and Methods

Plant Material

Plant material from the ethylene pathway mutant *etr1-1* (Bleecker et al., 1988) and *ctr1-2* (Kieber et al., 1993) was used for RNA extraction. Seeds were surface-sterilized and sown on Petri dishes with 1% (w/v) agar and half-strength Murashige & Skoog basal medium (pH5.65) with Gamborg's vitamins (MS media, Sigma). Seeds were stratified for 2 days at 4°C prior to exposure to the light. Green seedlings were grown at 22°C under constant illumination (45 $\mu E\ m^{-2}\ sec^{-1}$) for nine days after

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stratification. For growth of etiolated seedlings, seeds were light-treated for 12 hours prior to growth at 22°C in the dark for three and a half days.

RNA Isolation

Total RNA was extracted from 5 g of *Arabidopsis* seedlings following the protocol of Carpenter and Simon (Carpenter and Simon, 1998). mRNA was isolated from 0.5 - 1mg of total RNA using the PolyAtract mRNA Isolation System IV (Promega) following manufacturer's instructions. Fluorescent probe preparation was performed as described in Schenk et al. (2000)

Microarray hybridization (This part of the work was performed by Todd A. Richmond at Carnegie Institution of Washington.)

A set of 3,520 cDNA plasmid clones was used as the basis of the array. This set incorporated the 2,375 DNA sequences previously described in Schenk et al. (2000). In addition, a set of about 1,000 cDNA clones from root tissue was included along with various other clones. Here and after a "feature" is referred to a sequence present at a particular location on the array. Inserts were amplified by PCR, distributed into 384-well microtiter plates, and then spotted onto silylated microscope slides. Hybridization and washing steps were performed as previously described by Schenk et al. (2000).

Spot intensities from scanned slides were quantified using IMAGENE 2.0 software (Biodiscovery), and normalized by converting the fluorescence of each target spot into normalized values that are based on the standard deviation of the fluorescence of each channel as a whole (Student normalization; where the normalized value $S = \chi / \sigma$, χ = sample value and σ = standard deviation of the channel data; see Schenk et al., 2000). This has been proven to be a simple and robust method of normalization (Richmond and

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Somerville, 2000). Standard deviations were calculated based on three replicates from the same mRNA population. The background for each experiment was determined by using a set of control spots on the array. If the fluorescence of a target spot was greater than two standard deviations above the background for at least one channel, then that spot was considered as "above background". For detailed description of background, see Schenk et al, 2000. Positive ratios indicate induction in *ctr1-2* plants compared to *etr1-1* plants; negative ratios indicate repression.

Results and Discussion

General statistics

In this study, we examined the changes that occur in the abundance of transcripts corresponding to 3,520 *Arabidopsis* sequences. Data was generated using probes derived from mRNAs isolated from 4-d-old etiolated and 9-d-old green seedlings of *etr1-1* and *ctr1-2* mutants. The distinct phenotypes of *etr1-1* and *ctr1-2* mutants grown in the light or dark are shown in Figure 10. The etiolated *ctr1-2* seedlings display a typical "triple response": a short and radially-expanded hypocotyl, a pronounced apical hook, and a short primary root (Fig. 10A). This phenotype is drastically different from that of the *etr1-1* etiolated seedlings. In green seedlings, the difference in phenotype is not as pronounced as in etiolated seedlings. However, these two mutants are still easily distinguishable primarily due to the difference in length of the primary root (Fig. 10B). The use of two sets of mutants grown under different growth conditions is to find a set of genes that are regulated under both growth conditions. Those genes are more likely to be central to the ethylene signal transduction.

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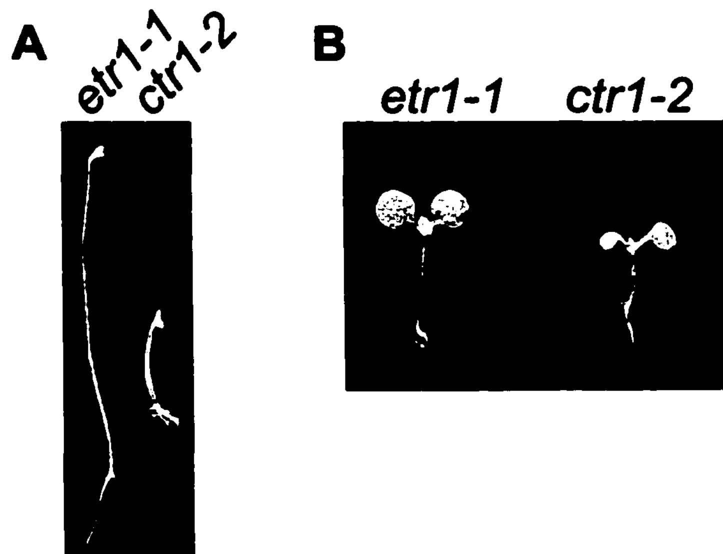

Figure 10. Phenotypes of *etr1-1* and *ctr1-2* mutants.
A. Images of 4-day-old *etr1-1* and *ctr1-2* etiolated seedlings.
B. Images of 9-day-old *etr1-1* and *ctr1-2* green seedlings grown under constant illuminatin.

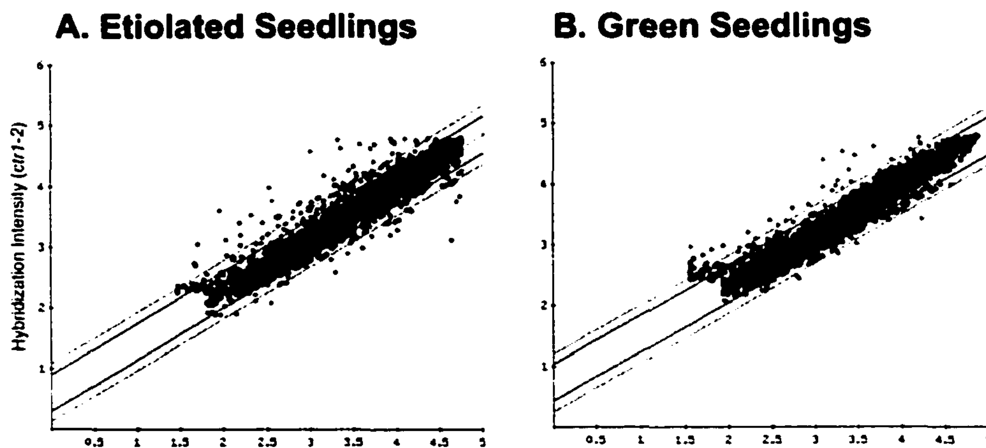

Figure 11. Scatter plot graphs of hybridization distribution patterns of 3,520 cDNAs on the microarray. Hybridizations were performed with labeled cDNA probes derived from mRNA of *ctr1-2* and *etr1-1* mutants (average of three replicates for each treatment). Guide lines represent 2-fold and 3-fold induction/repression ratio cutoffs were shown relative to the best fit line through the normalized data (middle line). A. Hybridizations using probes derived from etiolated seedlings. B. Hybridizations using probes derived from green seedlings.

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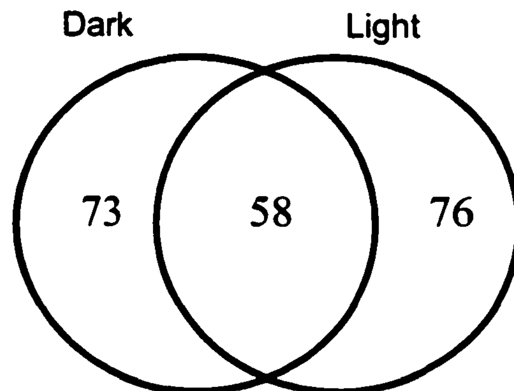
A Induced



B Repressed

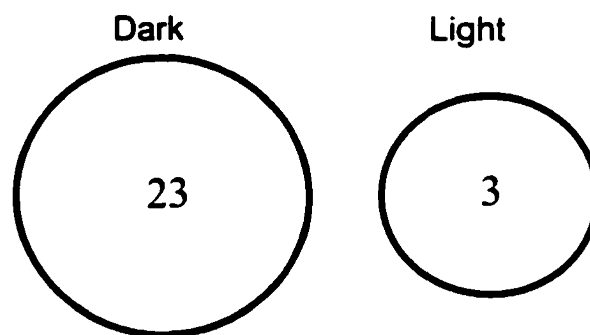


Figure 12. Venn diagrams of the numbers of induced or repressed genes on the microarray with ratios of at least 3.0.
A. Genes that showed induction under dark and light growth conditions overlap.
B. Genes that show repression under dark and light growth conditions do not overlap.

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In this analysis, a cutoff of 3 was used for the ratio of changes in expression level between *ctr1-2* and *etr1-1* (Fig. 11). With the etiolated seedlings, 156 features out of 3,520 made the ratio cutoff and were above background (4.7% of total features) (Fig. 11A). With the green seedlings, 139 features made the ratio cutoff and were above background (3.9% of total features) (Fig. 11B). Here and after, an induction indicates that a gene displays a higher expression level in *ctr1-2* compared to *etr1-1* and a repression indicates that a gene display a lower expression level in *ctr1-2* compared to *etr1-1*.

Under both growth conditions, the majority of features with ratios above the cutoff displayed an induction rather than a repression (Fig. 12). In etiolated seedlings, 24 of the features showed a reduction in gene expression whereas 131 showed an induction in gene expression. In green seedlings, this trend was even more pronounced, and only 4 of the features showed decreased expression but 134 of the features showed increased expression. Fifty-eight induced genes were shared by two growth conditions (overlapping). However, none of the repressed genes were shared by both growth conditions.

Genes that were induced under both growth conditions are shown in Table 1. A number of the genes display a higher induction in the dark than in the light. Examples of these genes are *ETR2* (At3g23150), *ERS2* (At1g04310), *ERS1* (At2g40940), an AP2-domain containing protein RAP2.3 (At3g16770), and an extensin (At4g12420). As shown in Figure 10, a dark-grown *ctr1-2* seedling has a short and radially-expanded hypocotyl, a pronounced apical hook, and a short primary root. In contrast, a dark-grown *etr1-1* seedling has a long, thin hypocotyl, a less pronounced apical hook, and a long

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primary root. In light-grown seedlings, the difference in phenotype between *ctr1-2* and *etr1-1* is not as pronounced as in dark-grown seedlings except for the difference in length of the primary root. Thus, ethylene has a greater visible effect upon the growth of dark-grown plants than upon light-grown plants, which may be reflected in more pronounced changes in gene expression in dark-grown plants.

Genes with the highest induction or repression level under either growth condition are shown in Table 2. There are two genes that demonstrated opposite regulation under the two different growth conditions: a plasma-membrane cell-wall linkage protein (At4g12550), which was repressed in the dark and induced in the light, and a chloroplast superoxide dismutase (At4g25100), which was induced in the dark and repressed in the light (Table 2). As shown in Figure 10A, dark-grown *etr1-1* and *ctr1-2* seedlings display pronounced difference in size of hypocotyls. This is likely to correlate with substantial differences in the synthesis of cell-wall components (Abeles et al., 1992). However, in light-grown seedlings, the difference between *etr1-1* and *ctr1-2* plants is not that pronounced, so there are less likely to be significant differences in the cell-wall. Therefore the expression of plasma-membrane cell-wall linkage protein is likely to be regulated differently in etiolated and green seedlings. The opposite regulation of chloroplast superoxide dismutase gene under the two growth conditions may result from the interaction of ethylene and the mechanisms needed to regulate photosynthesis. These would be different in dark- and light-grown seedlings.

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Table 1. Induced genes that are shared by two growth conditions

Description	Chromosome Locus	Genebank Accession	Ratio Dark	Ratio Light
Ethylene Receptor (ETR2)	At3g23150	AF047975	42.8	25.2
Ethylene Receptor (ERS2)	At1g04310	W43451	15.7	6.4
Ethylene Receptor (ERS1)	At2g40940	U21952	10.1	3.9
ethylene response sensor	At2g40940	N96844	7.5	5.3
ACC OXIDASE	At1g05010	R90435	6.3	3.5
AP1 (EcoRI fragment)	At1g69120	N/A	6.5	7.5
AP2 domain containing protein RAP2.3	At3g16770	R30178	27.3	16.4
AP2 domain containing protein RAP2.3	At3g16770	H37496	37.0	19.9
AP2 domain containing protein RAP2.3	At3g16770	T04320	12.9	10.0
AP2 domain containing protein RAP2.3	At3g16770	R90285	9.0	8.2
AP2 domain containing protein RAP2.2	At3g14230	N/A	3.1	3.1
LRR protein	At2g25790	F20107	3.3	3.9
cell death associated protein	At5g16080	#N/A	4.9	5.0
luminal binding protein	At5g28540	T20846	8.6	6.8
UFO(unusual floral organ)	At1g30950	N/A	4.7	6.8
AGL2 3' (EcoRI fragment)	At5g15800	N/A	7.0	8.8
putative phi-1-like phosphate-induced protein	At4g08950	N/A	6.7	5.2
peroxidase ATP14a	At5g22410	H36849	6.7	4.1
mitochondrial phosphate transporter	At3g48850	N/A	6.6	3.8
EXTENSIN	At4g12420	N38363	27.7	6.9
AtCslB2.cellulose synthase-like protein	N/A	AC004681	3.4	3.1
AtCslB4. cellulose synthase-like protein	N/A	AC004681	6.2	6.9
AtCSLB6. cellulose synthase-like protein	N/A	Z97338	4.8	5.9
putative NADH-ubiquinone oxireductase	At2g20800	N/A	10.4	4.0
epoxide hydrolases	At4g02340	T04215	18.6	5.7
cellulase	At3g44990	AA042790	9.8	3.1
SAG12,Cysteine protease	N/A	N/A	7.8	9.9
3-ketoacyl thiolase	At1g04710	AC002376	4.7	3.1
putative AAA-type ATPase	At2g18190	N/A	4.6	5.7
putative AAA-type ATPase	At2g18190	N/A	4.5	5.2
putative AAA-type ATPase	At2g18190	N/A	3.9	4.7
Histidine Kinase (T13L16.16)	At2g17800	AC003952	4.5	4.5
beta-glucosidase-like protein	At3g60120	N/A	6.8	6.6
pectate lyase	At5g04310	W43826	4.2	9.2
DFR (dihydroflavonol reductase)	At5g42800	N/A	3.8	14.7
RNA 3'-terminal phosphate cyclase	N/A	N/A	3.7	4.0
putative protein	At5g13210	N/A	6.6	3.9
hypothetical protein	At2g04070	N/A	3.2	3.3
hypothetical protein	At2g04050	N/A	15.9	3.4
hypothetical protein	At3g13600	N/A	4.5	4.7
unknown protein	At5g57770	N/A	8.7	8.1
unknown protein	At1g19180	N/A	4.9	4.2
GA-1	N/A	N/A	5.4	8.9
TAG	N/A	N/A	5.9	5.9

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dd-47GA-F(2)	N/A	N/A	3.7	5.1
no description	N/A	N/A	6.7	7.1
no description	N/A	N/A	6.5	6.1
no description	N/A	N/A	5.8	8.6
no description	N/A	AA041171	4.8	7.0
no description	N/A	N/A	4.4	4.8
no description	N/A	AA005667	4.1	3.3
no description	N/A	N/A	3.7	11.3
no description	N/A	N/A	3.6	4.9
no description	N/A	N/A	3.3	3.8
no description	N/A	N/A	3.3	3.2
no description	N/A	N/A	3.2	4.0
no description	N/A	N/A	3.2	12.6
no description	N/A	N/A	3.2	3.8

N/A: not accessible. Table 1. *Continued*

Table 2. Genes showing regulation dependent upon growth condition

Description	Chromosome Locus	Genebank Accession	Ratio Dark	Ratio Light
ACC OXIDASE	At1g62380	N96585	**6.4**	1.8
ACC OXIDASE	At1g77330	N/A	**4.7**	3.0
ethylene-responsive element binding protein	AT4g34410	N/A	**21.7**	2.7
beta-tonoplast intrinsic prote	At1g17810	R64952	**6.7**	1.4
A.thaliana rRNA repeat unit	X52322	N/A	**6.8**	1.2
AP2 domain transcription factor	AT3g25730	N/A	**6.8**	1.8
alpha-xylosidase precursor	At1g68560	R90271	**7.0**	2.8
cytochrome P450	At1g12740	N/A	**11.2**	2.8
ChoroplastpsbA	N/A	N/A	**12.0**	1.9
unknown protein	At3g20820	T22460	**6.3**	3.0
AGL2 5' (EcoRI fragment)	N/A	N/A	4.1*	**12.9**
AGL1 5' (EcoRI fragment)	N/A	N/A	3.5*	**12.9**
AGL15	N/A	N/A	2.8	**9.3**
mlo	N/A	N/A	1.1	**13.3**
antifreeze protein	N/A	F14159	4.5*	**10.8**
PR1(michigan)	N/A	N/A	4.8*	**13.3**
NBS/LRR disease resistance pro	At1g12280	Z17993	5.6*	**13.4**
resistance-like gene	N/A	N/A	5.5*	**14.5**
putative WRKY-type DNA binding protein	At2g38470	N/A	1.7	**8.0**
response regulator 6 (AB008489)	N/A	AA005467	-1.6	**9.3**
plasma membrane-cell wall link	At4g12550	H76464	**-34.7**	5.1
unknown protein	At2g17500	N/A	**-5.3**	-1.4
CelA04 (cellulose synthase)	At5g44030	T45893	**-8.2**	-1.5
nodulin-like protein	At1g75500	N/A	**-5.5**	-1.7
RNA helicase	N/A	N/A	**-5.3**	-1.5
major latex protein	At4g23670	T46481	**-9.4**	-1.8
superoxidase dismutase, chloroplast	At4g25100	AA042334	5.1	**-6.6**

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unknown protein	At1g70840	H36828	-2.3	**-3.1**
Drought-induced cysteine proteinase	At4g11320	T22938	-3.3*	**-3.1**
FERREDOXIN	At1g60950	H36817	-1.0	**-3.9**

+ : induction; - : repression; *: numbers with standard deviation >= 50%. Table 2. *Continued*
Numbers in bold indicate significant regulations. N/A: not accessible.

Functional classification of genes with induction

In an attempt to gain more information about the effect of ethylene at the physiological level, genes that showed an increase in gene expression were grouped into nine functional categories based on their annotations or their homologies to known genes (Fig.13). The genes that showed repression were not functionally classified because the number was not large enough to obtain a reasonable distribution. The nine categories are based on a previous classification of genes analyzed by microarray (Thimm et al., 2001), except that the signaling category is broken down into 3 categories: stress/defense signaling, ethylene signaling and other signaling. The nine functional categories are as follows: unknown/hypothetical (genes that are listed as hypothetical or unknown in the Genebank database), metabolism (amino acid, carbon, lipid, nucleic acid, nucleotide sugar and secondary metabolism, glycolysis, respiration), protein (protein modification and catabolism), transport (nutrient uptake and homeostasis), photosynthesis/chloroplast, cellular organization (cell wall and development, cytoskeleton, intracellular trafficking), stress/defense signaling, ethylene signaling, and other signaling (signal biosynthesis, signal perception and transduction, DNA-binding, RNA-binding, protein phosphorylation and dephosphorylation).

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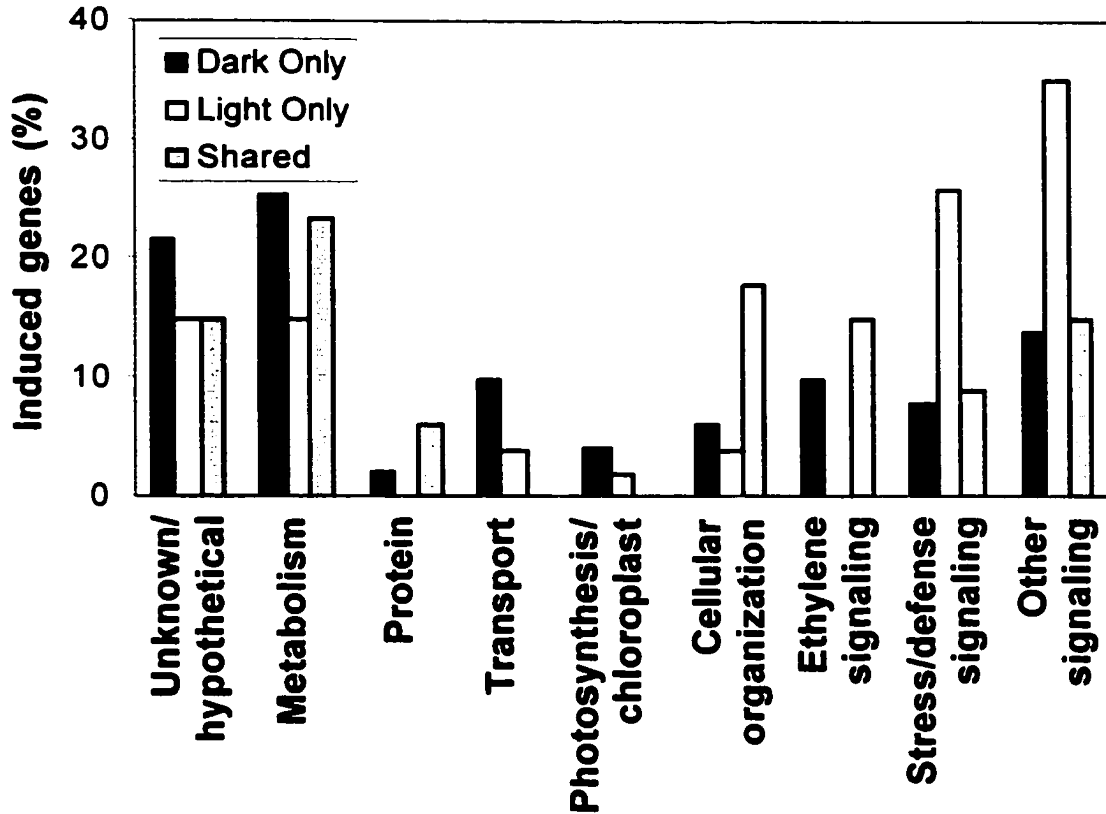

Figure 13. Functional distribution of induced genes. Ethylene-induced genes were classified into nine functional categories: unknown/hypothetical, metabolism (amino acid, carbon, lipid, nucleic acid, nucleotide sugar and secondary metabolism, glycolysis, respiration), protein (protein modification and catabolism), transport (nutrient uptake and homeostasis), photosynthesis/chloroplast, cellular organization (cell wall and development, cytoskeleton, intracellular trafficking), stress/defense, ethylene and other signaling (signal biosynthesis, signal perception and transduction, DNA-binding, RNA-binding, protein phosphorylation and dephosphorylation). Data is presented as three comparisons: genes induced only in the dark (dark only), genes induced only in the light (light only), and genes induced under both growth conditions (shared). Each bar represents the percetage of genes in that category out of the total nuber of induced genes under that growth condition. The total numbers of induced genes under dark, light and both growth conditions are 61, 61, and 45, respectively.

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The distribution of the induced genes in these functional categories was analyzed using: (1) genes that are only induced under the dark growth conditions, (2) genes that are only induced under the light growth conditions, and (3) genes that are induced under both growth conditions.

The distribution demonstrated that the number of induced genes in each category varies. A large number of genes are involved in signaling, which includes stress/defense signaling, ethylene signaling and other signaling. A detailed analysis of genes in each category follows. Emphasis is given to genes that are regulated under both dark and light growth conditions because these genes are more likely to represent genes that perform a consistent and central role in ethylene signaling and responses.

1. Induced genes with unknown/hypothetical functions

The twenty-four genes in this category were described as those encoding uncharacterized proteins. Of these, 11 genes showed induction only in the dark; whereas 8 genes showed induction only in the light. The remaining 5 genes were induced under both conditions.

2. Induced genes involved in metabolism

Genes involved in metabolism comprises 21% (twenty-nine genes) of the total number of genes analyzed. Eight genes in this category were induced in both the light and dark, one of which encodes an enzyme involved in primary metabolism (3-ketoacyl thiolase (At1g04710)) and another one an enzyme involved in secondary metabolism (dihydroflavonol reductase (At5g42800)). Ethylene is known to regulate metabolism during fruit-ripening in tomato (Zegzouti et al., 1999). However, in this analysis, the

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Arabidopsis seedlings used had not reached the fruit-forming stage. Thus, the data supports a role for ethylene in modulating metabolism during growth and development in *Arabidopsis*.

3. Induced genes involved in proiein modification and catabolism

Three genes were found to be involved in protein modification and catabolism, with two of these induced in both the light and dark. These two genes encode an AAA-type ATPase (At2g18190), which is involved in endocytosis (Babst et al., 1998), and the senescence-inducible SAG12 cysteine protease (Gan and Amasino, 1995). The *Arabidopsis* seedlings used in this analysis were not senescent. Therefore it is likely that the SAG12 promoter is activated in response to ethylene rather than strictly in response to a senescence-associated signal. The induction of the cysteine protease may be involved in ethylene-associated protein turnover.

4. Induced genes involved in transport

Seven genes analyzed fell into the nutrient uptake and homeostasis category. Five of these genes were induced in the dark; two were induced in the light. No gene in this category was induced in both the light and dark, suggesting that genes in this category may be not be central to plants' ethylene responses but dependent upon the growth condition instead.

5. Induced genes involved in photosynthesis

Three of the induced genes are involved in photosynthesis. Two of these are induced in the dark; one gene is induced in the light. None was induced in both the light and dark. This suggests that other mechanisms besides ethylene signal transduction are involved in regulating ethylene's effect upon induction of photosynthetic genes.

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6. Induced genes involved in cellular organization

There are a total of 11 genes in this category. Six genes were induced in both the light and dark. These encode enzymes associated with cell-wall synthesis and hydrolysis, such as AtCslB2, and AtCslB4 (cellulose synthase-like), and cellulase (At3g44990). Ethylene is known to alter the synthesis and composition of the cell-wall (Abeles et al., 1992). Ethylene increases the thickness of cell-wall, changes the pattern of cellulose fibri deposition, and increases the accumulation of cell-wall hydroxyproline, which makes cell-wall inextensible. Cellulase has the potential for changing cell-wall thickness and/or structure (Abeles et al., 1992). The induction of AtCslBs and cellulase may be associated with a difference in cell-wall structures of *ctr1-2* and *etr1-1* due to the effect of constitutive ethylene response in the *ctr1-2* mutant and ethylene insensitivity in the *etr1-1* mutant.

7. Induced genes involved in ethylene signaling

Within this category, all ten of the genes induced in the light were also induced in the dark. Therefore there are no genes in the category for those that are only induced in the light. A number of genes that are involved in ethylene biosynthesis and ethylene signal transduction were induced in both the light and dark. Several of the genes encode ACC oxidases (At1g77330, At1g62380, At1g05010), a key enzyme in the ethylene biosynthesis pathway (Yang and Hoffman, 1984). Previous studies had suggested that ACC oxidases may play an important role in regulating ethylene biosynthesis, especially during conditions of high ethylene production (Schaller and Kieber, 2002). ACC oxidase catalyzes the last step in the ethylene biosynthetic pathway, the ethylene-forming step.

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The induction of ACC oxidase potentially allows for higher ethylene production by the plant, as ethylene biosynthesis would not be limited by the availability of this enzyme.

The ethylene receptors ERS1 (At2g40940), ERS2 (At1g04310), and ETR2 (At3g23150) were also induced. Such induction of ethylene receptors had been previously demonstrated by means of Northern blot analysis using ethylene treatment (Hua et al., 1998). Interestingly, the ethylene receptor gene *ETR1* was also slightly induced with its induction level right around the cutoff ratio: 3.3-fold in the dark and 2.9-fold in the light. *ETR1* was not listed in Table 1 as its induction level in the light is below the cutoff ratio used. A slight 1.4-fold induction of *ETR1* by ethylene was previously observed using Northern blot analysis (Hua et al., 1998). The level of induction based on microarray analysis may not directly correlate with Northern blot analysis due to different normalization methods used for the two analyses. The induction of the ethylene receptor genes by the hormone ethylene may re-sensitize the plant to ethylene. The half-life for ethylene to dissociate from its receptor ETR1 is 12.5 hours (Schaller and Bleecker, 1995). Such a slow dissociation rate would not allow the ethylene-bound receptors to release ethylene quickly enough to perceive changes in ethylene concentration. Thus, the induction of ethylene receptor genes by ethylene would produce new "empty" receptors that could sense if ethylene is still present.

Several transcription factors previously demonstrated to be involved in ethylene signaling were also induced by ethylene. The transcription factor AP2-domain containing protein RAP2.2 (At3g14230), which interacts with ethylene response element (ERE) present in the promoters of ethylene inducible genes (Riechmann and Meyerowitz, 1998), was induced in both the light and dark. The induction of the AP2-domain

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containing protein gene by ethylene had also been observed in a previous microarray analysis (Schenk et al., 2000).

8. Induced genes involved in stress/defense signaling

Twenty-one genes were involved in stress/defense signaling. Fourteen of these genes are induced only in the light, including genes encoding disease resistance proteins (At3g50950, At1g72890), a heat-shock transcription factor (At3g51910), PR1 (Pathogenesis Related protein 1) (At2g19990) (Uknes et al., 1992), a glutathione S-transferase (At2g02930), and a WRKY-type DNA-binding protein (At2g38470), a transcription factor involved in stress responses (Eulgem et al., 2000). Genes that are induced in the dark encode proteins such as the nematode-resistance protein (At2g40000), wound-induced protein 1 (At3g10980) and a salt-tolerance zinc finger protein (At1g27730). The three genes that are induced in both the light and dark are a LRR (Leucine Rich Repeat) protein gene (At2g25790), a cell death associated protein gene (At5g16080) and a luminal binding protein gene (At5g28540). As ethylene mediates stress and defense responses in plants (Pennickx et al., 1996; O'Donnell et al., 1996; Roman et al., 1995; Alonso et al., 1999), the induction of stress/defense-associated genes in my microarray experiment is expected.

9. Induced genes involved in other signaling

Genes for signaling that are not known to be involved in ethylene signaling and stress/defense signaling are grouped as "other signaling". Thirty-one of the induced genes belong to this category. Genes that were induced in both the light and dark are *AP1* (*APETALA 1*) /*AGL7* (*AGAMOUS-like*) (At1g69120), a histidine kinase gene (At2g17800), *UFO* (unusual floral organ) (At1g30950), *AGL2* (*AGAMOUS-like*)

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(At5g28540). In addition to *AGL2* and *AGL7*, several other *AGL* genes (*AGL1*, *AGL4*, *AGL8*, *AGL11*, and *AGL15*) were also strongly induced under the light growth conditions. *AGL* genes are transcription factors involved in determining floral organ identity (Taiz and Zeiger, 1998). However, the seedlings used in this study had not reached flowering stage, thus my data suggest that the *AGL* genes may be involved in other uncharacterized aspects of growth and development. Other genes that are induced only in the light are genes encoding several protein kinases (At3g25250, At3g09010, At5g35370), receptor-protein kinases (At2g25790, At2g27060), response regulators (ATRR1 (At1g10470), ATRR6 (At5g62920)), SAUR (small-auxin-upregulated-RNA) (At4g38850), a pollen-specific protein (At4g12420) and a RING zinc finger protein (At1g74370). The genes that are induced only in the dark include genes for the DNA-binding protein CCA1, which is involved in circadian rhythms (Mizoguchi et al., 2002), a Myb-family transcription factor, and a protein phosphatase 2C ABI1 (At4g26080), which is involved in abscisic acid signaling (Bonetta and McCourt, 1998). These results are consistent with the interaction by ethylene signaling with other signaling pathways (Beaudoin et al., 2000; Ghassemian et al., 2000; Rao et al., 2000). It is also possible that some of these genes may be specific for ethylene signaling, but had not previously been identified as such.

Identification of putative ethylene-response elements in promoters of ethylene-receptor genes

A region 1.5-kb upstream from the start methionine in each of the ethylene receptor genes was analyzed for putative ethylene response promoter elements. The ethylene receptor genes *ETR2*, *ERS2*, and *ERS1*, which all showed strong induction in

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both light and dark (Table 1), have sequences in their promoter regions that are similar to a previously identified promoter element, known as the primary ethylene response element (PERE). The PERE element was not observed in the promoter region of the ethylene receptor genes *ETR1* and *EIN4* that are not strongly induced by ethylene. This promoter element was originally found in the promoter region of the *Ethylene-Response-Factor1* (*ERF1*) gene as an imperfect palindromic structure which specifically binds to EIN3 (Solano et al., 1998). A similar motif was also found in the promoter regions required for ethylene responsiveness in other ethylene-inducible genes such as the tomato *E4* (Montgomery et al., 1993) and the carnation *GST1* genes (Itzhaki et al., 1994; Solano et al., 1998). An alignment of the known PEREs from the *Arabidopsis ERF1* gene, the tomato *E4* gene, and the carnation *GST1* gene with the putative PEREs from the ethylene receptor genes *ETR2*, *ERS2*, and *ERS1* is shown in Figure 14. A degenerate palindromic structure is shared by all these genes. However, variations such as insertions and deletions in this palindromic element are observed for the different genes as observed before by Solano et al. (1998). I propose that this imperfect palindromic element may serve as an ethylene-inducible promoter element for ethylene receptor genes *ERS1*, *ERS2*, and *ETR2* and that *ERS1*, *ERS2*, and *ETR2* may be primary targets for transcriptional regulation by ethylene signal transduction. Confirmation of the role of these putative primary ethylene response elements in the ethylene induction of *ERS1*, *ERS2* and *ETR2* awaits more experimentation.

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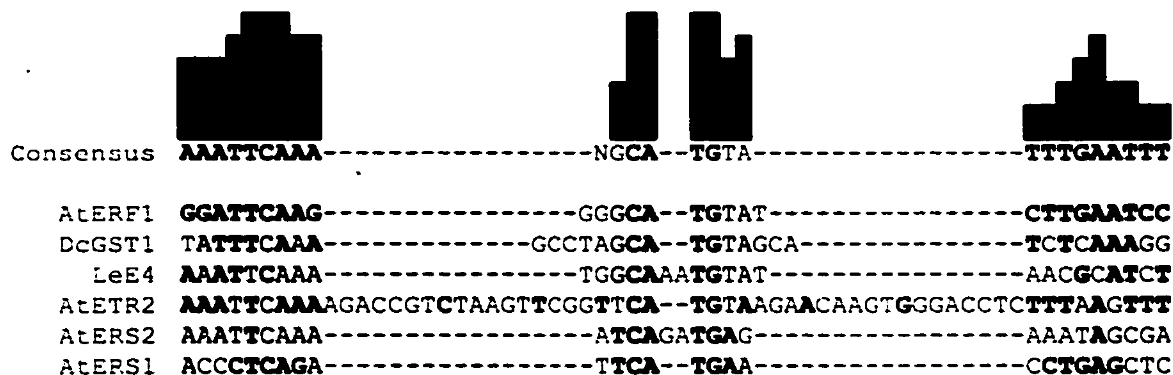

Figure 14. Alignment of known ethylene response elements with the proposed ones of the ethylene receptors. Known ethylene response elements from *Arabidopsis* (AtERF1), tomato (LeE4) and carnation (DcGST1) were shown. Nucleotides in common with the consensus were shaded in grey. The black bars above the consensus indicate the number of nucleotides that all sequences have in common with the consensus. Nucleotides that form an internal palindromic repeat were in bold.

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Conclusion

Reciprocal mutants of the ethylene signal transduction pathway were employed in analysis of gene expression by microarray. Comparison of gene expression in the constitutive ethylene response mutant *ctr1-2* relative to the ethylene-insensitive mutant *etr1-1* should mimic a maximal effect of ethylene upon plant gene expression. In this study, 235 out of 3,520 genes showed significant differential expression in the *ctr1-2* mutant compared to the *etr1-1* mutant. Among these genes, 207 genes were induced (expression level was higher in *ctr1-2* than in *etr1-1*), and 28 genes were repressed (expression level was lower in *ctr1-2* than in *etr1-1*). Schenk et al. performed a similar microarray analysis with 2,375 genes using 24-hr ethylene-treatment of 200 μL/L on adult *Arabidopsis* plants (8~12 leaf stage). They obtained 137 ethylene-responsive genes, with 117 genes induced and 20 genes repressed, when a cutoff ratio of 2.5 was applied (Schenk et al., 2000). The use of reciprocal mutants in this study gave similar results to ethylene treatment used by Schenk et al. (2000).

The use of reciprocal mutants for microarray analysis thus represents a powerful approach by which to study gene expression in plants. It should be particularly useful for the analysis of genes that have a low threshold for induction or repression by endogenous plant regulators. Regulation of such genes is difficult to detect by conventional methods that involve external delivery of the regulator to the plant. However, the limitation on such a mutant-based approach is that one can only examine long-term, not transitory effects upon regulation.

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A close look at the regulated genes provides us with more information about the effect of ethylene on gene expression. The ethylene receptor gene *ETR1* was induced by 3.3-fold under the dark growth conditions and 2.9-fold under the light growth conditions. A 1.4-fold induction of *ETR1* has been previously observed by Northern blot analysis using ethylene-treated plant tissues (Hua et al., 1998). The use of reciprocal mutants and microarray analysis therefore appears to allow for detection of subtle inductions by ethylene.

The ethylene receptor genes *ETR2*, *ERS2*, and *ERS1* displayed 42.8-, 15.7-, and 10.1-fold induction under dark growth conditions, respectively. Under light growth conditions, the induction was 25.2-, 6.4-, and 3.9-fold, respectively (Table1). The induction level observed by northern blot using the ethylene-treatment method was about 6-fold for *ETR2*, *ERS2* and *ERS1* (Hua et al., 1998). Using a similar microarray employed for this analysis, Schenk et al observed a 4.3-fold induction for *ERS2*; significant changes in expression of ETR2 and ERS1 could not be determined due to insufficient dada from their microarray. Analysis of reciprocal mutants by microarray may have resulted in larger induction ratios than previously seen when using ethylene for the following reasons: (1) the background induction caused by endogenous ethylene can not be eliminated by means of ethylene treatment, (2) the ethylene treatment was not long enough for some genes to reach their maximum induction, (3) the concentration of ethylene used for treatment was not optimum for induction, and (4) normalization methods used for microarray and Northern blot analysis differ.

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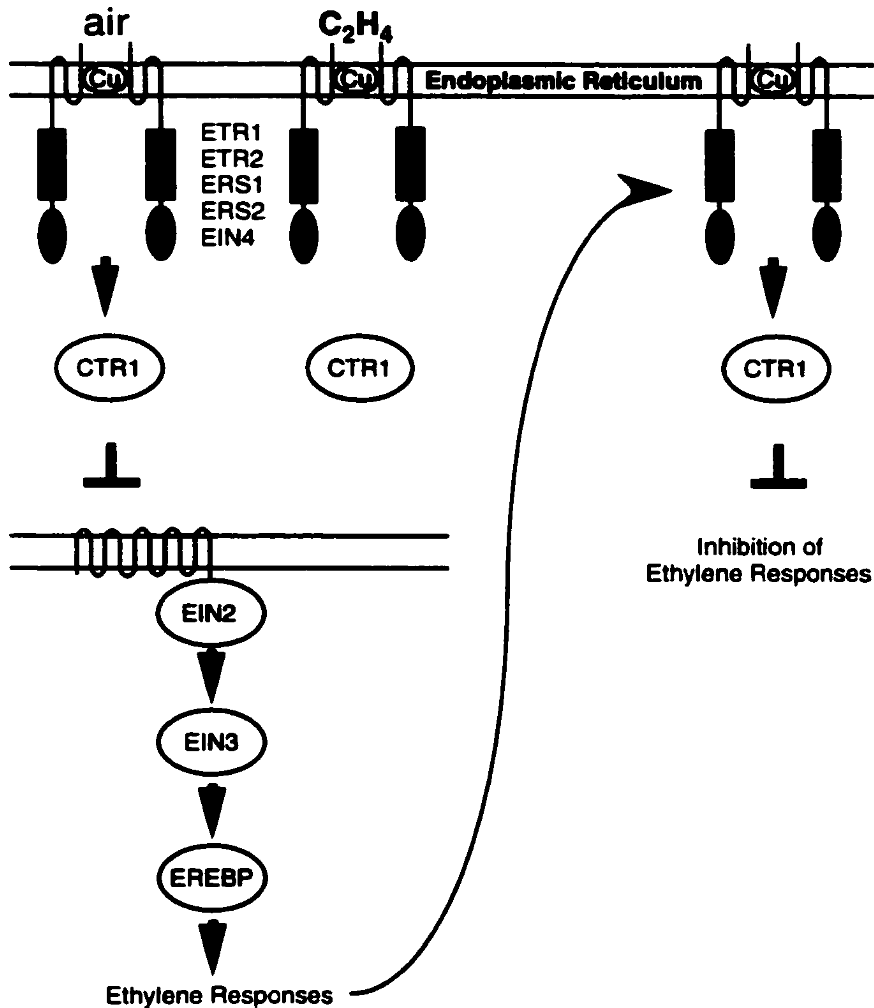

Figure 15. A model for induction of ethylene receptors by ethylene. In air, ethylene receptors are in active forms, and ethylene responses are repressed. In the presence of ethylene, ethylene receptors bind to ethylene and turn into an inactive form. The repression on ethylene response is released. The ethylene-induced biosynthesis of new "empty" receptors can account for the re-sensitization of the plant to ethylene. If ethylene levels have decreased, the newly synthesized receptors will not bind ethylene and repress ethylene responses in the plant. If ethylene is still present, the newly synthesized receptors will bind ethylene and unable to repress ethylene responses.

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The induction of ethylene receptor genes *ETR2*, *ERS1* and *ERS2* by ethylene can be explained by the model shown in Figure 15. In air, ethylene receptors are thought to activate the downstream component CTR1, which is a negative regulator of ethylene responses. Therefore actions downstream of CTR1 are arrested, and ethylene responses are repressed. In the presence of ethylene, ethylene receptors bind to ethylene and no longer activate CTR1; this relieves the repression on downstream signal transduction and results in the induction of ethylene responses. If the receptors have all bound ethylene, the plant would not be able to detect a reduction in ethylene levels due to the slow dissociation rate of ethylene from its receptors. However, the ethylene-induced biosynthesis of new "empty" receptors can account for the re-sensitization of the plant to ethylene. If ethylene levels have decreased, the newly synthesized receptors will not bind ethylene and will therefore repress ethylene responses in the plant. If ethylene is still present, newly synthesized receptors will bind ethylene and be unable to repress ethylene responses.

An examination of ethylene-induced gene expression indicates the physiological effects of ethylene are wide-ranging and complicated. Some of the ethylene effects may depend on factors such as tissue type, growth conditions, and developmental stage. The purpose of using plants grown under two completely different growth conditions in this study was to identify genes that are regulated by ethylene similarly under both growth conditions. The genes that display similar regulation by ethylene independent of the growth condition are more likely to be central to ethylene responses in the plant. Genes that are involved in metabolism, protein degradation, cell-wall synthesis and hydrolysis, ethylene signaling, stress/defense signaling and other signaling are induced under both

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growth conditions. Some of these genes had not been previously identified as ethylene-regulated genes.

In summary, my results indicate that many genes, especially those implicated in signaling, are regulated by ethylene. A number of genes that are involved in ethylene biosynthesis or ethylene signal transduction are induced by ethylene. Expression of the ethylene receptor genes *ETR2*, *ERS1* and *ERS2* are significantly induced by ethylene. The expression of *ETR1* is induced to a limited extent. The expression of *EIN4* did not show appreciable changes in response to ethylene. My results also indicate that the use of reciprocal mutants for microarray analysis provides an effective approach to study gene expression in plants.

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CHAPTER IV

EFFECT OF SALT AND OSMOTIC STRESS UPON EXPRESSION OF ETHYLENE RECEPTORS

(This chapter is based on a manuscript prepared for submission: Zhao, X-C., Richmond, T.A., Schaller, G.E. Regulation of Ethylene Receptor Expression in *Arabidopsis thaliana* by Salt and Osmotic Stress. The microarray hybridizations were performed by Todd A. Richmond at the Carnegie Institution of Washington.)

Introduction

The phytohormone ethylene is a key regulator of plant growth and development. Ethylene promotes seed germination, seedling growth, leaf and petal abscission, fruit ripening, and organ senescence (Abeles et al., 1992). Ethylene is also involved in the response of the plant to abiotic stresses such as pathogen attack and abiotic stresses such as freezing, wounding, and drought (Roman et al., 1995; O'Donnell et al., 1996; Penninckx et al., 1996). Extensive research has demonstrated that ethylene biosynthesis is modulated by numerous factors including biotic and abiotic stresses (Yang and Hoffman, 1984; Kende, 1993). For example, both hypo-osmotic and hyperosmotic shock have been shown to induce 1-aminocyclopropane-1-carboxylate (ACC) synthase, the key enzyme involved in regulating ethylene biosynthesis (Felix et al., 2000).

In *Arabidopsis*, ethylene is perceived by a five-member family of receptors: ETR1, ETR2, ERS1, ERS2 and EIN4 (Hua et al., 1995; Hua et al., 1998; Sakai et al., 1998). These contain amino-terminal transmembrane domains that encompass the

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ethylene-binding site, ethylene binding having been demonstrated for ETR1 and ERS1 (Schaller and Bleecker, 1995; Hall et al., 2000). The ethylene receptor ETR1 localizes to the endoplasmic reticulum of *Arabidopsis* (Chen et al., 2001). In the carboxyl-terminal half, the receptors contain regions with homology to histidine kinases and in some cases the receiver domains of response regulators (Schaller, 2000; Chang and Stadler, 2001). These are signaling elements originally identified as parts of bacterial two-component systems that are now known to also exist in plants, fungi, and slime molds (Parkinson, 1993; Ota and Varshavsky, 1993; Chang et al., 1993; Swanson et al., 1994). Histidine kinase activity has been confirmed for ETR1 (Gamble et al., 1998), but its role in ethylene signal transduction is still unclear (Gamble et al., 2002).

Hormonal signal transduction can be regulated at several levels by varying the concentration of hormone, receptor, or other downstream components. Although a number of factors have been demonstrated to regulate ethylene levels in the plant (Yang and Hoffman, 1984; Kende, 1993), only limited information is available on the regulation of ethylene receptor levels owing to their relatively recent identification. The one factor previously demonstrated to affect the expression of ethylene receptors is ethylene itself, which has been shown to induce the expression of *ETR2*, *ERS1*, and *ERS2* in *Arabidopsis* (Hua et al., 1998). In this chapter, it is shown that the expression of the ethylene receptors *ETR1*, *ETR2*, and *ERS1* is down-regulated by salt and osmotic stress. Thus abiotic stresses, in addition to regulating ethylene signal transduction by modulating hormone levels, may also do so by modulating receptor levels.

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Materials and Methods

Plant Material and Growth Conditions

Arabidopsis thaliana (ecotype Columbia) was grown as follows: seeds were surface-sterilized and sown on Petri dishes with 1% (w/v) agar and half-strength Murashige & Skoog basal medium (pH5.65 - 5.85) with Gamborg's vitamins (MS media, Sigma). Seeds were stratified for 2 days at 4°C prior to growth at 22°C.

For expression analysis of genes involved in the salt-stress response, seeds were placed on sterile filter paper on top of the MS agar medium (Savouré et al., 1997), and grown for 7 days at 22°C under continuous illumination (45 $\mu E\ m^{-2}\ sec^{-1}$) with cool-white fluorescent lights. Filter papers with seedlings were then transferred to 100 x 20 mm Petri dishes containing 10 mL liquid MS medium with either 0 mM (control) or 200 mM (salt-treated) NaCl. Control plants were grown on medium for 24 hr. Salt-treated plants were grown on medium for either 2 or 24 hr, as indicated.

For analysis of ETR1 protein levels receiving salt or osmotic stress, seedlings were grown on filter paper as described above, then transferred to 10 mL liquid MS medium containing NaCl, $NaNO_3$, KCl, mannitol, or sorbitol at concentrations and for incubation times as indicated in the figure legends.

Germination Assays

The degree of sensitivity to osmotic-adjusting chemicals was examined on half-strength medium supplemented with varying concentrations of NaCl, $NaNO_3$, KCl, mannitol, or sorbitol as indicated in the figure legends. After stratification, seeds were kept under continuous illumination for 7 days. Germination was then scored. Standard deviations are shown for a minimum of 40 seeds per treatment from at least 3 separate

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experiments. Seeds were not considered germinated until their cotyledons turned green (Quesada et al., 1999).

Isolation of mRNA

Total RNA was extracted from 5 g of *Arabidopsis* seedlings following the protocol of Carpenter and Simon (Carpenter and Simon, 1998). mRNA was isolated from 0.5 - 1mg of total RNA by use of the PolyAtract mRNA Isolation System IV (Promega) following manufacturer's instructions.

Microarray hybridization (This part of the work was performed by Todd A. Richmond at the Carnegie Institution of Washington.)

Construction of the microarrays, preparation of probes, hybridization, and washing steps were done as previously described (Schenk et al., 2000). Spot intensities from the scanned slides were quantified using the ImaGene 2.0 software (Biodiscovery), and normalized by converting the fluorescence of each target spot into a normalized value that is based on the standard deviation of the fluorescence of each channel as a whole (Student normalization; where the normalized value $S = \chi / \sigma$, χ = sample value and σ = standard deviation of the channel data; see Schenk et al., 2000). This has proved to be a simple and robust method of normalization (Richmond and Somerville, 2000). Standard deviations were calculated based on three replicates from the same mRNA population. Positive ratios indicate induction by treatment; negative ratios indicate repression.

Northern Blot Analysis

Aliquots of the same mRNA samples used for microarray analysis were used for Northern blot analysis. mRNA was separated on 1% agarose gels containing glyoxal and

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dimethylsulfoxide using the NorthernMax-Gly kit (Ambion) according to the manufacturer's instructions. mRNA was then transferred to a nylon membrane (Millipore) by the capillary method and fixed by UV cross-linking. Hybridizations were performed using buffers supplied with the NorthernMax-Gly kit. Single-stranded anti-sense probes were made using primers designed to anneal at the 3' end of the selected genes. Probes were made and the blot stripped between hybridizations by the use of Strip-EZ PCR kit (Ambion) according to the manufacturer's instructions. Radioactivity was imaged and quantitated by phosphor-imaging with a Molecular Imager FX (BioRad) and Quantity One software.

Protein Isolation and Immunoblot Analysis

For isolation of *Arabidopsis* membranes, plant materials were homogenized at 4°C in extraction buffer (30mM Tris-HCl, pH 8.5, 150mM NaCl, 10mM EDTA, 20% glycerol) containing 1mM phenylmethylsulfonyl fluoride, 1µg/mL pepstatin, 10µg/mL aprotinin, and 10µg/mL leupeptin as protease inhibitors. The homogenate was strained through Miracloth (Calbiochem) and centrifuged at 8,000 x g for 15min. The supernatant was centrifuged at 100,000 x g for 30min, and the membrane pellet then resuspended in resuspension buffer (10mM Tris, pH 7.5, 150mM NaCl, 1mM EDTA, 10% glycerol) with same protease inhibitors at used for homogenization. Protein concentration was determined by a modified version of the Lowry assay (Schaller and DeWitt, 1995). Bovine serum albumin was used as a standard.

For immunoblot analysis, membranes were mixed with SDS-PAGE loading buffer containing 100mM of dithiothreitol and incubated at 37°C for 1hr. Proteins were fractionated by SDS-PAGE using 8% (w/v) polyacrylamide gels. After electrophoresis,

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proteins were electrotransferred to PVDF Immobilon membrane (Millipore). Western blotting was performed by using anti-ETR1 (401-738), or anti-H^+- ATPase, or anti-BiP polyclonal antibodies. Anti-ETR1(401-738) was prepared against a GST-fusion protein with amino acids 401-738 of ETR1 (Schaller et al., 1995). The anti-H^+-ATPase antibody (DeWitt et al., 1996) was provided by Dr. M. Sussman (University of Wisconsin, Madison). The anti-BiP antibody (Höfte and Chrispeels, 1992) was provided by Dr. M. Chrispeels (University of California, San Diego). Protein bands were visualized by enhanced chemiluminescence detection according to the manufacturer (Pierce). Densitometric analysis was performed using NIH Image (Version 1.60) after first scanning the exposed film and then capturing the image with Adobe Photoshop. Relative levels of the immunodecorated ETR1 were determined by comparison to a dilution series of ETR1.

Results

Expression of *ETR1*, *ERS1*, and *ETR2* is repressed by salt-treatment

Microarray experiments were used to examine the salt-stress response in green seedlings (Fig. 16). Gene expression was analyzed in seedlings treated for 2-hour or 24-hour with 200 mM NaCl, a level of salt that has been demonstrated previously to lead to stress responses and changes in gene expression in *Arabidopsis* (Peng et al., 1996). For each comparison, 3 independent hybridization experiments were performed. Ratios were determined and averaged. In this analysis, only changes in mRNA abundance in excess of 3-fold that of control (non-treated) were considered. With the 2-hr treatment, 62 out of 2,375 features (about 2.6% of total features) made a ratio cutoff of 3 and were above

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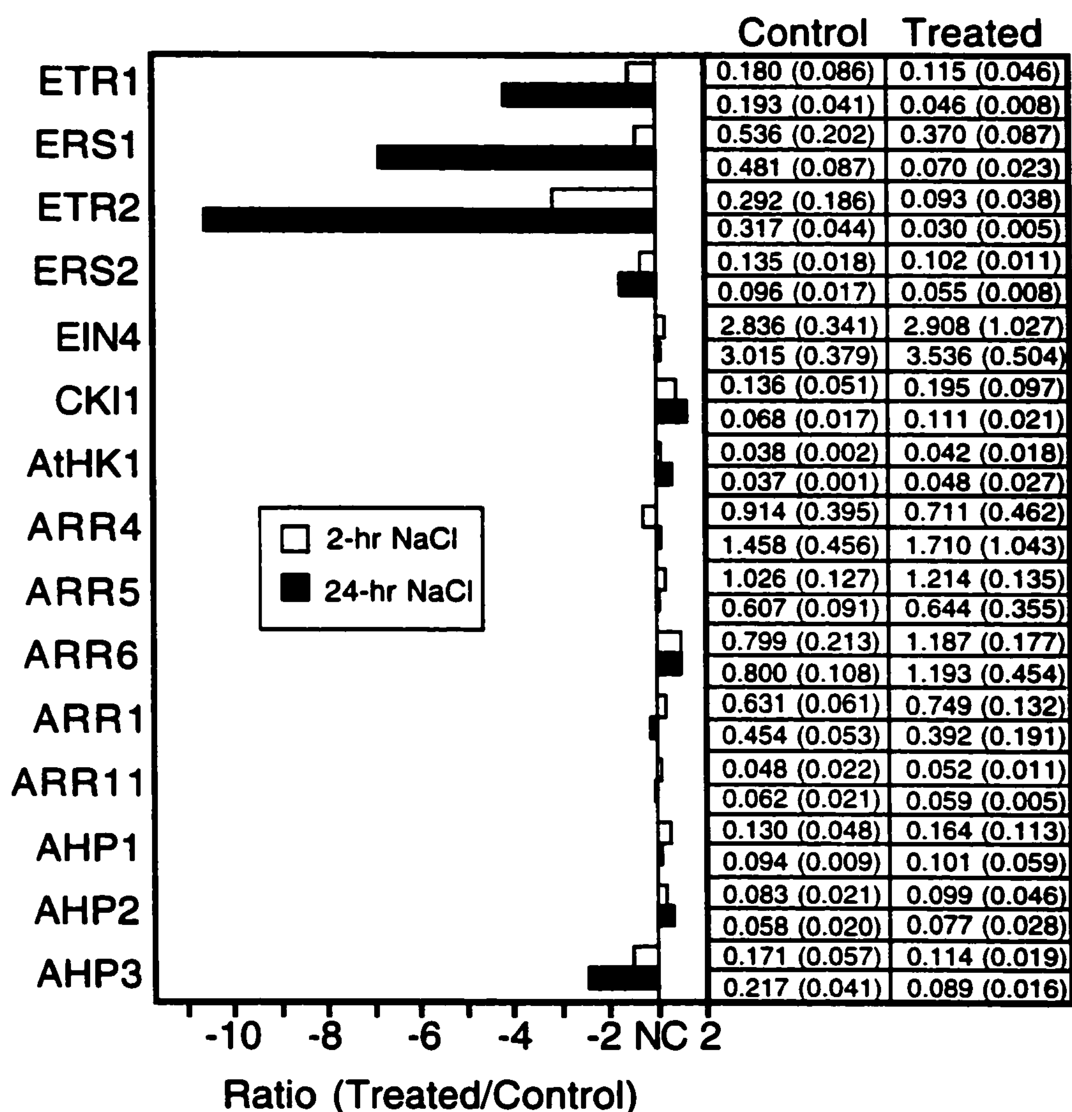

	Control	Treated
ETR1	0.180 (0.086)	0.115 (0.046)
	0.193 (0.041)	0.046 (0.008)
ERS1	0.536 (0.202)	0.370 (0.087)
	0.481 (0.087)	0.070 (0.023)
ETR2	0.292 (0.186)	0.093 (0.038)
	0.317 (0.044)	0.030 (0.005)
ERS2	0.135 (0.018)	0.102 (0.011)
	0.096 (0.017)	0.055 (0.008)
EIN4	2.836 (0.341)	2.908 (1.027)
	3.015 (0.379)	3.536 (0.504)
CKI1	0.136 (0.051)	0.195 (0.097)
	0.068 (0.017)	0.111 (0.021)
AtHK1	0.038 (0.002)	0.042 (0.018)
	0.037 (0.001)	0.048 (0.027)
ARR4	0.914 (0.395)	0.711 (0.462)
	1.458 (0.456)	1.710 (1.043)
ARR5	1.026 (0.127)	1.214 (0.135)
	0.607 (0.091)	0.644 (0.355)
ARR6	0.799 (0.213)	1.187 (0.177)
	0.800 (0.108)	1.193 (0.454)
ARR1	0.631 (0.061)	0.749 (0.132)
	0.454 (0.053)	0.392 (0.191)
ARR11	0.048 (0.022)	0.052 (0.011)
	0.062 (0.021)	0.059 (0.005)
AHP1	0.130 (0.048)	0.164 (0.113)
	0.094 (0.009)	0.101 (0.059)
AHP2	0.083 (0.021)	0.099 (0.046)
	0.058 (0.020)	0.077 (0.028)
AHP3	0.171 (0.057)	0.114 (0.019)
	0.217 (0.041)	0.089 (0.016)

Figure 16. Microarray analysis of the salt-stress effect upon expression of two-component signaling elements. Seven-day-old wildtype green seedlings were treated with MS media (control), or 200mM NaCl/MS solution for either 2 hrs or 24 hrs. The bars represent the change in expression level of genes encoding two-component signaling elements from treated plants versus control plants. A positive number indicates a higher expression level in salt-treated plants than in control plants. A negative number indicates a lower expression level in salt-treated plants than in control plants. The mean hybridization intensity of each gene is given based on three replicates, with the standard deviations shown in parentheses. NC -- no change.

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background. With the 24-hr treatment, 481 features made a ratio cutoff of 3 and were above background (about 20.2% of total features).

Features on the array included DNA sequences for all five members of the ethylene receptor family: *ETR1*, *ERS1*, *ETR2*, *ERS2*, and *EIN4* (Chang et al., 1993; Hua et al., 1995; Hua et al., 1998; Sakai et al., 1998). Also represented on the array were DNA sequences for additional histidine kinases (*CKI1* and *AtHK1*), response regulators of both the type-A (*ARR4*, *ARR5*, and *ARR6*) and type-B (*ARR1* and *ARR11*) classes, and histidine-containing phosphotransfer proteins (*AHP1*, *AHP2*, and *AHP3*). These, like the ethylene receptors, are two-component signaling elements found in *Arabidopsis* and may participate together in multi-step phospho-relays (Schaller, 2000). These clones were confirmed by sequencing to contain only the sequence of interest and the results reported here are based on these known sequences.

Some members of the ethylene receptor family exhibited a reduction in expression level following exposure of seedlings to 200 mM NaCl (Fig .16). *ETR2* showed the most pronounced changes, displaying a 3.1-fold reduction in expression after 2-hr treatment and a 10.6-fold reduction in expression after 24-hr treatment. *ETR1* and *ERS1* also showed pronounced changes in expression, with 4.2-fold and 6.8-fold reductions after 24-hr treatment, respectively. Only minimal changes in expression levels were found for *ERS2* and *EIN4*. Of the other genes examined representing two-component signaling elements, only *AHP3* showed a significant change in expression, in a case, a reduction of 2.4-fold after 24-hr salt treatment.

The same mRNA samples used for microarray analysis were also used for Northern blot analysis (Fig.17). Northern blot analysis was performed to examine

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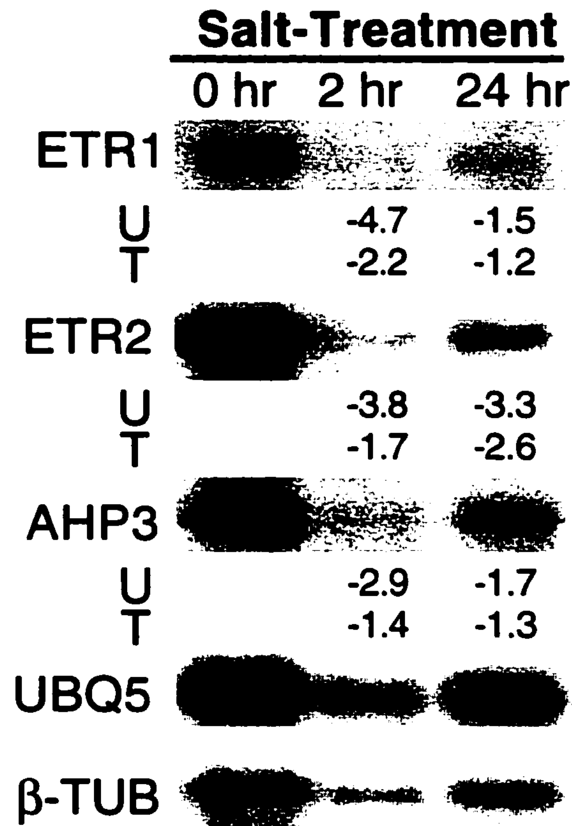

Figure 17. Northern blot analysis of selected genes for two-component signaling elements. The same mRNA populations used for microarray analysis were used for Northern blot analysis. RNA blots were probed for the two-component regulators *ETR2*, *ETR1*, and *AHP3*. *UBQ5* and β-tubulin were used as control probes. The numbers below lanes represent the levels of expression after normalization to either *UBQ5* (U) or *β-tubulin* (T). Comparison is to the controls, each of which is assigned to a value of 1.0. Northern blots were quantitated with a Biorad Phospho-Imager using Quantity One software.

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expression of the genes *ETR1*, *ETR2*, and *AHP3*, all of which showed changes in expression under one or both of the conditions examined by microarray analysis. Two different genes, the ubiquitin extension protein gene *UBQ5*, and the β-tubulin gene, were used as controls for normalization. Genes required for ubiquitination and the cytoskeleton are frequently referenced in the plant literature as controls for Northern blot analysis as their expression is assumed to remain relatively constant (Taniguchi et al., 1998; Vogel et al., 1998; Espinosa-Ruiz et al., 1999; Shi et al., 2000). Reduction in expression level of *ETR1*, *ETR2* and *AHP3* was also observed by Northern blot analysis after normalization to the control gene expression (Fig. 17). However, results obtained using β-tubulin for normalization were not consistent with those obtained using UBQ5.

Repression of ETR1 expression upon salt treatment is confirmed at the protein level

To determine if the reduction in transcript level of the ethylene receptors resulted in a reduction in protein level, membranes were isolated from green seedlings exposed to 200 mM NaCl for various lengths of time and the protein level of ETR1 determined by immunoblot analysis (Fig.18A). The protein levels of ETR1 did not show any appreciable change through 36 hr of NaCl treatment. However, after 48-hr treatment, the level of ETR1 was reduced to approximately half of its original level. After 72-hr treatment, the level of ETR1 was reduced still further. The loss of membrane proteins was not a general feature of the salt-stress response in *Arabidopsis*. When analyzed by SDS-PAGE, many membrane proteins showed little change in abundance over the entire 72 hr of salt treatment. In addition, immunoblot analysis revealed that neither the plasma membrane H^+-ATPase nor BiP, a resident protein of the endoplasmic reticulum, showed

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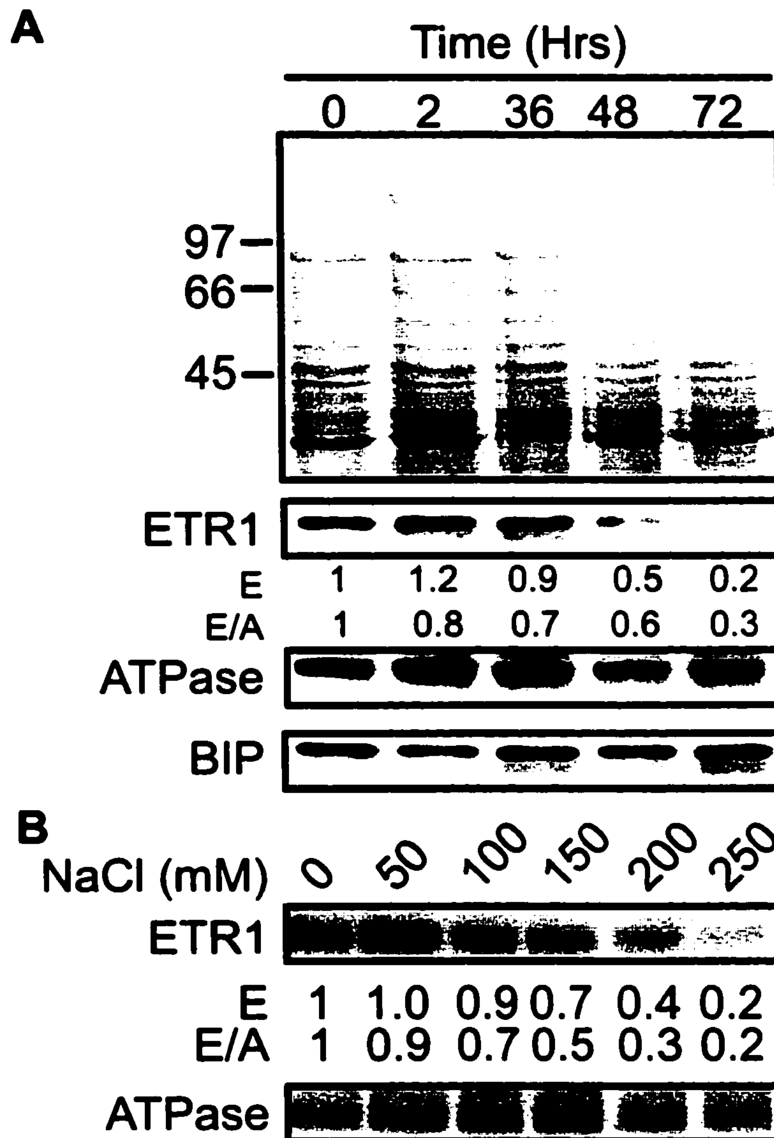

Figure 18. Effect of NaCl-treatment upon ETR1 expression. A, Time course for the effect of salt stress upon protein levels of ETR1. Membrane proteins were isolated from 7-day-old green wild-type seedlings receiving 2, 36, 48, and 72 hours of salt-treatment (200mM NaCl). Coomassie-stained SDS polyacrylamide gel profiles of 50 μg membrane proteins are shown from each time point. Migration positions of molecular weight markers are indicated in kDa. Specific membrane proteins were visualized by immunoblot using antibodies against ETR1, H^+-ATPase, and BiP. The apparent molecular masses of H^+-ATPase and BiP as determined from SDS-PAGE and immunoblot were 100 kDa and 77 kDa, respectively. The expression level of ETR1 was determined by densitometric quantification of immunoblots and is normalized against expression in the control (E), and is also normalized against levels of H^+-ATPase (E/A). B, Dose dependence of ETR1 expression of plants treated for 72-hr with different concentrations of NaCl.

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significant changes in abundance after 72 hr of salt treatment (Fig. 18A). Examination of the sensitivity of ETR1 expression to varying levels of NaCl (Fig. 18B) indicated that effects of NaCl could be distinguished after 72-hr treatment with 100 to 150 mM NaCl. The results indicate that the ETR1 ethylene receptor is reduced at the protein level, consistent with the reduction in mRNA level revealed by microarray analysis.

Expression of ETR1 is also repressed upon treatment with other osmoticums

To determine whether the effects of NaCl treatment were due to ion toxicity and/or osmotic stress (Zhu, 2001), the effects of additional salts as well as mannitol and sorbitol upon expression of ETR1 were examined. Mannitol and sorbitol are non-ionic osmoticums that are commonly used to examine the effects of osmotic stress on plants (Zamski et al., 2001; Lew, 1996).

Wildtype seedlings were treated with two other ionic osmoticums, $NaNO_3$ and KCl, over the same concentration range previously examined with NaCl. After treatment of seedlings with $NaNO_3$ or KCl for 72 hr, the protein level of ETR1 was determined. Immunoblot analysis showed that the protein level of ETR1 exhibited a similar trend of decrease after treatment with $NaNO_3$ or KCl for 72 hr (Fig. 19A, B). In both cases, the ETR1 level exhibited an obvious decrease when the concentration of salts reached 100 - 150 mM, similar to the results observed with NaCl treatment. The results suggest that the ionic toxicity contributed to the reduction in ETR1 level.

To investigate the expression of ETR1 following osmotic stress, seedlings were treated with mannitol and sorbitol for 72 hr and examined for their ETR1 protein level (Fig. 20A, B). The concentration range of mannitol and sorbitol used here showed

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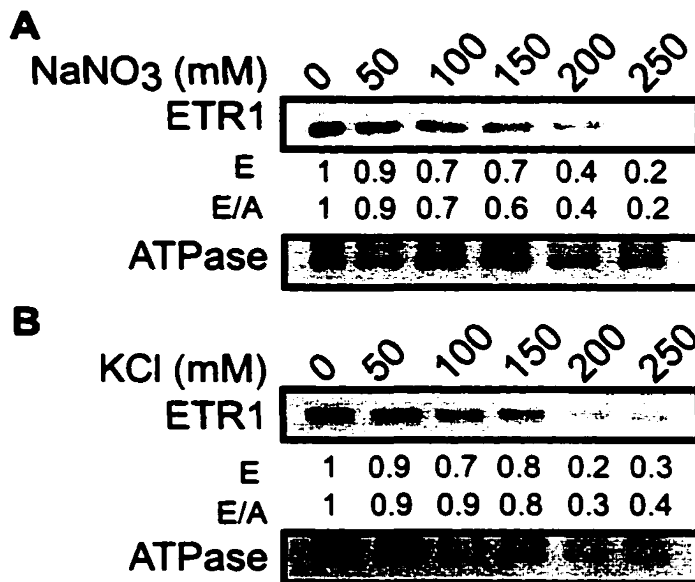

Figure 19. Effect of $NaNO_3$ and KCl-treatment upon ETR1 expression. 7-d-old wildtype green seedlings were treated with different concentrations of $NaNO_3$ (A), and KCl (B) for 72 h. Expression levels were quantified densitometrically (E) and also normalized against immunologically determined levels of the H^+-ATPase (E/A) as an internal control.

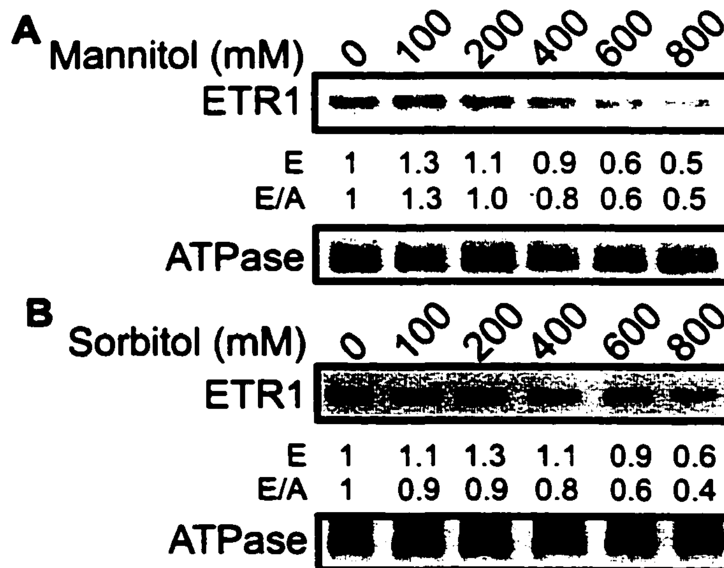

Figure 20. Effect of mannitol and sorbitol-treatment upon ETR1 expression. 7-d-old wildtype green seedlings were treated with different concentrations of mannitol (A), and sorbitol (B) for 72 h. Expression levels were quantified densitometrically (E) and also normalized against immunologically determined levels of the H^+-ATPase (E/A) as an internal control.

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similar osmotic pressures to those obtained from the salt treatments (Werner et al., 1995). The ETR1 protein level was found to decrease as the concentration of osmoticums increases (Fig. 20A, B), consistent with an effect of osmotic stress upon the expression of ETR1. However, the reduction in ETR1 was not as pronounced as that observed with ionic osmoticums. The result suggests that the reduction in ETR1 may result from a combination of both salt and osmotic stress upon the plant.

Ethylene pathway mutants displayed different resistance to salt and osmotic stress

To further investigate whether the ethylene response is required for plants to sustain salt and osmotic stress, ethylene pathway mutants were tested for their sensitivity to salt and osmotic stress (Fig. 21). Two ethylene-insensitive mutants (*etr1-1* and *ein2*) and one constitutive ethylene response mutant (*ctr1-2*) were examined for their germination and growth in response to various salts and osmotic stresses.

Seeds of wildtype, *etr1-1*, *ein2* and *ctr1-2* were sown on media supplemented with osmoticums at the same concentration range used for above treatments. Germination and growth was examined 7 days after exposure to the light. Only those seedlings displaying green cotyledons were considered to have germinated.

When germinated on NaCl-containing media (Fig. 21A), the germination rate of *etr1-1* and *ein2* dropped rapidly with the increase of NaCl concentration and ceased to germinate at 150 mM NaCl. Wildtype did not show a significant reduction in germination rate until the concentration reached 150 mM, but no longer germinated at 200 mM NaCl. The germination rate of *ctr1-2* remained above 90% from 0 to 200 mM NaCl, but did not germinate at 250 mM NaCl. These results demonstrate that *ctr1-2* is

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more resistant to NaCl than wildtype, while *etr1-1* and *ein2* are more sensitive to NaCl than wildtype.

Similar effects were observed when seeds were germinated on media containing other osmoticums (Fig. 21B, C, D, E). In response to other osmoticums, the *etr1-1* and *ein2* mutants were also more sensitive than wildtype, and the *ctr1-2* mutant was more resistant than wildtype. These results indicate that the constitutive ethylene response mutant *ctr1-2* is much more resistant to the effect of salt and osmotic stress upon germination than ethylene-insensitive mutants.

The morphology of seedlings germinated on media containing different concentrations of NaCl was examined (Fig. 22). Because the mutants responded similarly to different osmoticums, only seedlings grown on NaCl-supplemented media are shown. With the increase of NaCl concentration, all seedlings germinated displayed a gradual reduction in size. However, the level of reduction was different. At 0 mM NaCl, the *etr1-1* and *ein2* mutants and wildtype plants were not distinguishable in terms of size. At 100 mM NaCl, *etr1-1* and *ein2* seedlings were significantly reduced in cotyledon size, hypocotyl length, and root length. But same level of reduction was not observed with wildtype plants until NaCl concentration reached 150 mM. Due to its constitutive-ethylene-response phenotype, the *ctr1-2* mutant plant is always reduced in size, and cannot be directly compared to wildtype. However, one can still see the effect of NaCl on its size, although it is not as obvious as for *etr1-1*, *ein2* and wildtype. The results are consistent with the constitutive ethylene response mutant *ctr1-2* being more resistant to salt and osmotic stress than wildtype, while ethylene-insensitive mutants *etr1-1* and *ein2* are more sensitive to salt and osmotic stress than wildtype.

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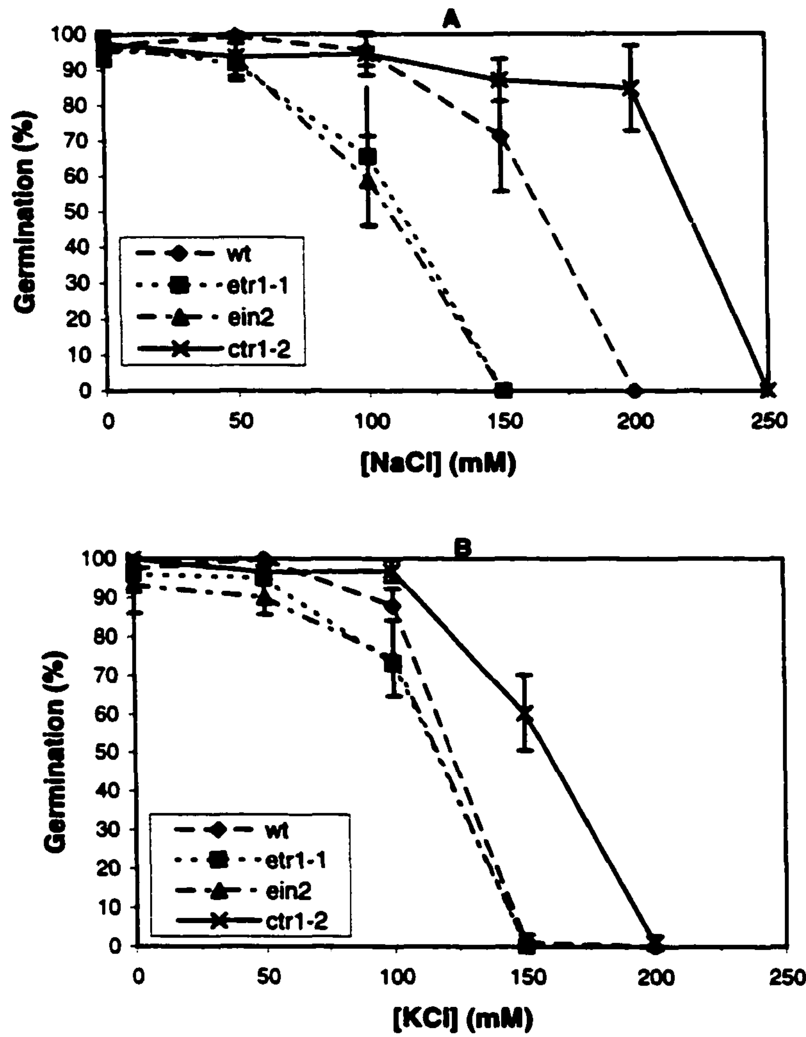

Figure 21. Sensitivity of ethylene pathway mutants to various osmoticums. Seeds of ethylene pathway mutants *etr1-1, ein2, and ctr1-2* as well as wildtype were sown on half-strength MS media containing varying concentrations of NaCl (A), NaNO3 (B), KCl (C), mannitol (D), and sorbitol (E). Germination was scored 7 days after exposing to the light. Each point corresponds to the average percentage germination of three or more replicates of at least 40 seeds. Error bars represent standard deviation.

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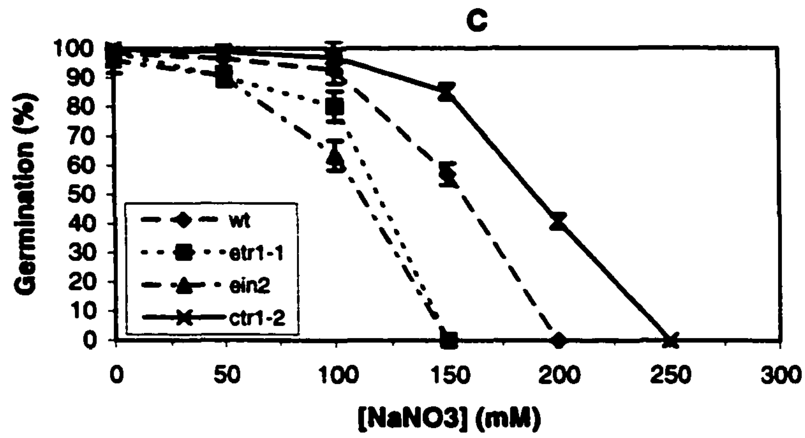

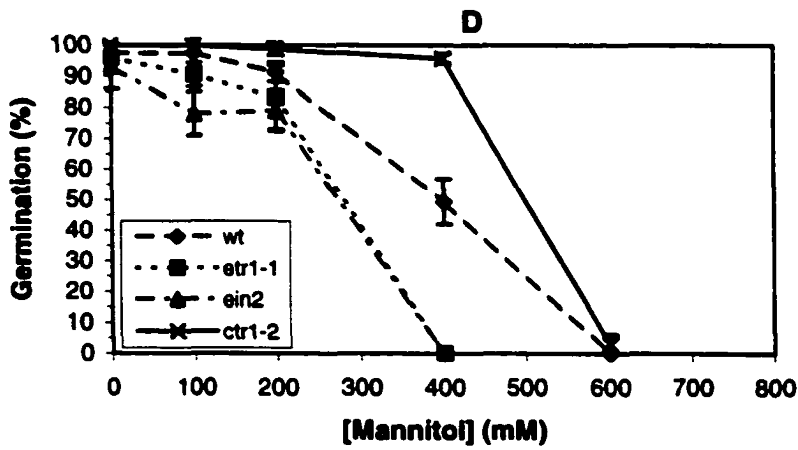

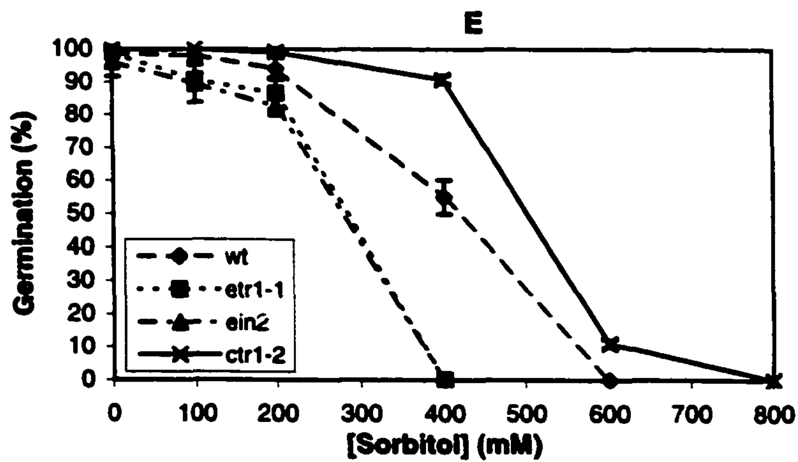

Figure 21. *Continued*.

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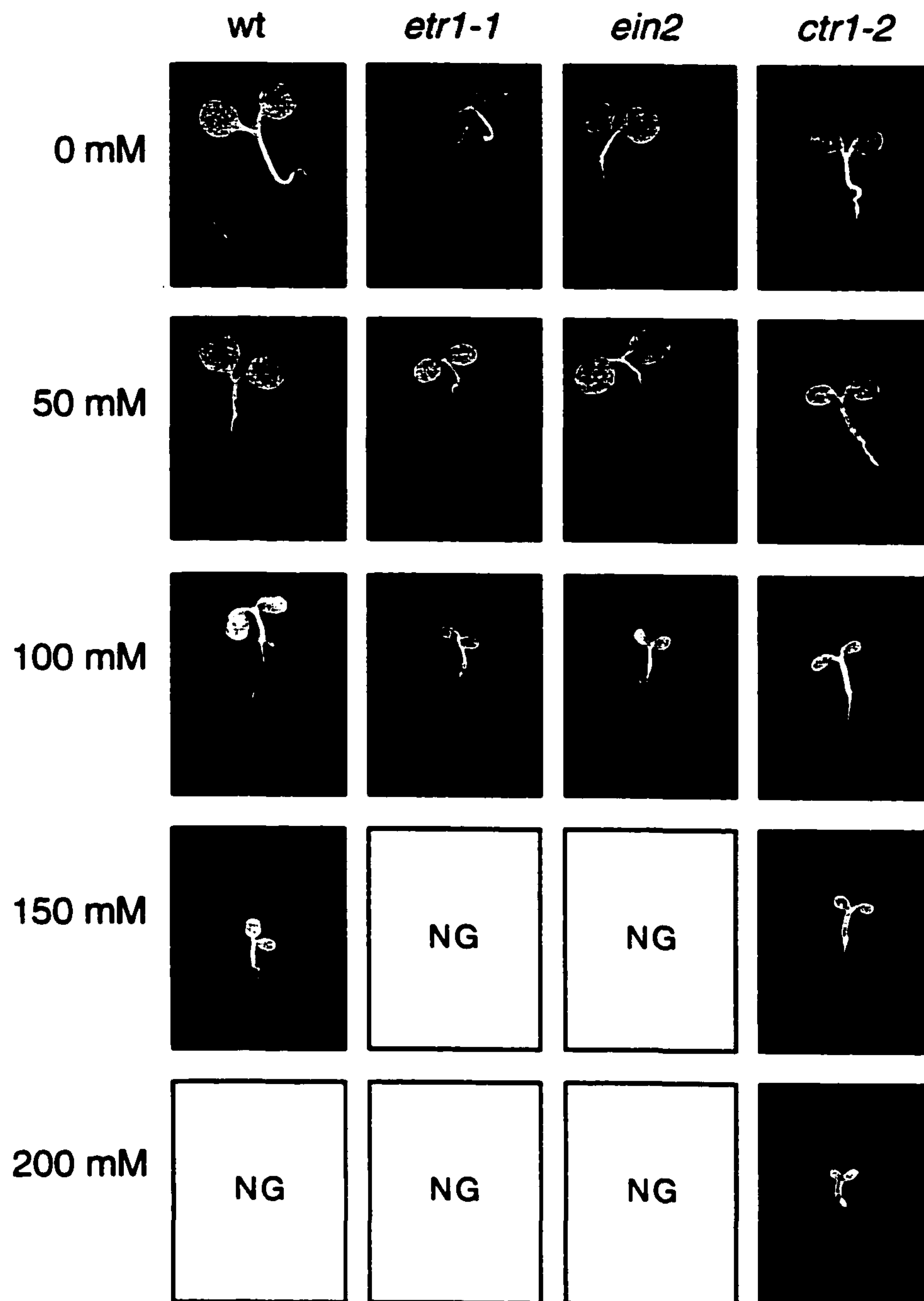

Figure 22. Morphology of seedlings grown on NaCl-containing media. Seeds were plated as described in Figure 21 and representative seedlings photographed after 7 days under continuous illumination. No germination is indicated by NG.

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Discussion

Ethylene receptors serve as negative regulators of the ethylene signal transduction pathway (Hua and Meyerowitz, 1998). Thus a decrease in receptors is predicted to induce ethylene responses, even without an ethylene stimulus, if the receptors are reduced sufficiently in number. It is also possible, dependent upon the level of reduction in ethylene receptors, for the plant to be sensitized such that a lower level of ethylene is sufficient to induce a response. These predictions have been confirmed experimentally. Loss-of-function mutants have been isolated in four out of five members of the ethylene receptor family in *Arabidopsis* (Hua and Meyerowitz, 1998). Individually, the loss-of-function mutants have little or no apparent effect on ethylene responses. However, triple and quadruple mutants of these genes display constitutive ethylene response phenotypes in the absence of ethylene, indicating that a reduction in receptor number can induce ethylene responses. Results obtained from tomato by taking an antisense-based approach to decrease the levels of ethylene receptors also indicate that receptors negatively regulate ethylene signal transduction (Tieman et al., 2000). Reduced expression level of a single ethylene receptor, *LeETR4*, mimicked such ethylene responses as fruit ripening, flower senescence, and leaf epinasty. Etiolated seedlings of *LeETR4* antisense lines also displayed a reduction in seedling growth at lower levels of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid than wildtype seedlings, indicating a greater sensitivity to ethylene in the mutant lines.

The ethylene receptors *ETR2*, *ETR1*, and *ERS1* showed reduced expression levels when *Arabidopsis* seedlings were subjected to salt and osmotic stress. Based on microarray analysis, the level of reduction varied from 4.2-fold for *ETR1* to 10.6-fold for

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ETR2 after 24-hr salt treatment. An examination of ETR1 protein levels indicated that the reduction at the message level was reflected at the protein level but with a significant lag. A decrease in ETR1 protein levels was first observed following 48-hr salt treatment. This lag could arise from a requirement for the ETR1 message to fall below a threshold level before protein levels are affected or be due to a slow rate of turnover for the ETR1 protein. In support of the second hypothesis, we have observed a slow turnover rate for ETR1 in *Arabidopsis* plants treated with the protein biosynthesis inhibitor cycloheximide (Chen, Randlett, and Schaller, unpublished results).

The reduction in transcript levels observed with the Northern blot analysis varied with that from the microarray analysis. For example, microarray analysis indicated that ETR2 was repressed over 10-fold after 24-hr salt treatment, while Northern blot indicated only a 1.5 or 2-fold reduction in expression dependent on what gene was used for normalization. The difference is likely to result from the different normalization methods used in Northern blot and microarray analysis. With Northern blot analysis, expression of the gene of interest was normalized to that of a single control gene. Thus Northern blot analysis was reliant upon the selected control gene maintaining a constant expression level. With microarray analysis, expression of the gene of interest was normalized to the total level of expression of all the genes on the microarray. Thus normalization was unaffected by changes in expression level of individual genes, and instead relied upon the overall level of gene expression remaining relatively constant. Moreover, according to our unpublished microarray data, expression levels of many genes were significantly repressed by salt-treatment. The expression of *β-tubulin* (Genebank accession no. T13823, clone no. 41F10T7) decreased 1.2-fold after 2-hr salt-treatment and 3.7-fold

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after 24-hr salt treatment. Therefore, in this case, using the expression of other genes as controls for normalization could not generate an accurate result.

The reduction in receptor levels should make the plant more sensitive to ethylene and could lead to activation of ethylene responses independent of the presence of ethylene. The discovery that ethylene receptors decrease in number during salt and osmotic stress is therefore consistent with the activation of ethylene responses constituting a component of the plant stress response. Given the lag observed between the initial exposure of plants to salt-tress and the reduction in ethylene receptors at both the transcript and protein levels, activation of ethylene signal transduction by this mechanism would be a secondary but potentially important response to salt and osmotic stress. Several steps lead to the activation of ethylene responses during salt and osmotic stress. (1) Perception of a change in salt and osmotic levels and signal transduction through a primary response pathway. (2) Reduction in transcription of ethylene receptors and/or increased turnover of mRNA for ethylene receptors. (3) Decrease in ethylene receptors at the protein level resulting in activation of the ethylene signal transduction pathway. Thus, reduction in receptor levels could serve as a mechanism for maintaining or activating ethylene responses in the absence of hormone.

In hormone perception, varying the concentrations of hormone, receptor, or downstream signaling elements can modulate signal transduction. Many experiments have demonstrated that ethylene biosynthesis is regulated by environmental stresses and other plant hormones (Abeles et al., 1992; Woeste et al., 1999), and that levels of ethylene vary in different plant tissues and at different developmental stages (Yang and Hoffman, 1984). It is now becoming clear that levels of the ethylene receptors are also

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dynamically regulated thereby providing another means by which ethylene signal transduction can be activated and repressed.

The analysis of mutant *Arabidopsis* plants is consistent with the activation of the ethylene signal transduction pathway being required for the responses to salt and osmotic stress. Both the ability to germinate and the growth of the seedlings following germination were affected in the mutants exposed to salt and osmotic stress. The ethylene-insensitive mutants were more sensitive to salt and osmotic stress than wildtype plants, but a constitutive ethylene response mutant was more resistant to salt and osmotic stress than wildtype plants. It is already known that ethylene protects plants from abiotic stresses such as freezing, wounding and flooding (Roman et al., 1995; O'Donnell et al., 1996; Penninckx et al., 1996). The expression level of 1-aminocycloproane-1-carboxylate (ACC) synthase, the key enzyme involved in regulating ethylene biosynthesis, is induced by both hypo-osmotic and hyperosmotic shock (Felix et al., 2000). My results taking a mutant-based approach are consistent with ethylene signal transduction being a component of plant resistance to salt and osmotic stress. Abscisic acid (ABA) is demonstrated to mediate osmotic stress (Xiong et al, 1999). *EIN2*, a central component of ethylene signaling pathway, is believed to be involved in responses to abscisic acid (Ghassemian et al., 2000). The ethylene signaling pathway was shown to inhibit ABA signaling in seeds (Beaudoin et al., 2000). A study on ozone-induced cell death in *Arabidopsis* also suggested that cross-talk between salicylic acid (SA), jasmonic acid (JA), and ethylene-dependent signaling pathways regulates plant responses to both abiotic and biotic stress factors (Rao et al., 2000). It is likely that the resistance to salt

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and osmotic stress requires the interaction of ethylene signaling with other hormonal pathways such as ABA and SA.

In summary, the results described here indicate that the ethylene receptors ETR2, ETR1, and ERS1 are down-regulated by salt and osmotic stress at transcriptional level. A reduction at the protein level was also observed with ETR1 but with a significant lag. The decrease in ETR1 may result from a combination of both salt and osmotic stress upon the plant. The reduction in receptor level may serve as a mechanism to activate ethylene signal transduction in response to salt and osmotic stress. Ethylene may be required for plants to sustain salt and osmotic stress as the constitutive-ethylene-response mutant was much more resistant to salt and osmotic stress than the ethylene-insensitive mutants.

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CHAPTER V

SUMMARY

Conclusions of this Dissertation

Ethylene is a plant hormone that has profound effects upon plant growth and development (chapter I). In *Arabidopsis*, ethylene is perceived by a five-member family of receptors: ETR1, ETR2, ERS1, ERS2, and EIN4. Understanding the regulation of ethylene signal transduction will not only enrich our knowledge about hormonal signaling in plants, but also provide potential means for manipulating the ethylene response in plants of agronomic value. Hormonal signal transduction can be regulated by varying the concentration of hormone, receptor, or other downstream components. A substantial amount of research has been performed to study the modulation of ethylene biosynthesis in plants. However, the regulation of ethylene receptor levels has not been extensively studied.

The focus of this dissertation is on the regulation of ethylene receptor levels in *Arabidopsis*, which I hypothesize is a potential mechanism to control ethylene responses in plants. I took three different approaches to study the regulation of ethylene receptor levels in *Arabidopsis*. In the first approach, I used various mutants in the ethylene signaling pathway to study the effect of the mutations upon expression of the ethylene receptor ETR1 (chapter II). Second, I used a reciprocal-mutant-based microarray analysis to investigate gene expression regulated by ethylene (chapter III). Third, I

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examined the dynamic changes in expression levels of ethylene receptors in response to abiotic stress (chapter IV). A summary of the results from my studies is shown in Figure 23.

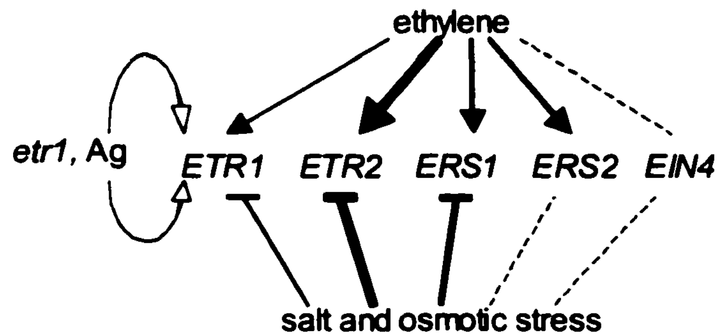

Figure 23. Regulation of ethylene receptor expression in *Arabidopsis* revealed by this research. The expression of *ETR2*, *ERS1* and *ERS2* were strongly induced by ethylene. The strongest induction was observed for *ETR2*. The expression of *ETR1* was induced to a limited extent. The expression of *EIN4* was not significantly affected by ethylene. The expression of *ETR1*, *ETR2* and *ERS1* was repressed by salt and osmotic stress. The strongest repression was observed for *ETR2*. The expression of *ERS2* and *EIN4* was not significantly regulated by salt and osmotic stress. Ethylene-insensitive mutation *etr1* can increase ETR1 protein level in a post-transcriptional manner. This effect can be mimicked by silver treatment.

To study the effect of various mutations upon expression of the ethylene receptor ETR1, ETR1 protein levels were quantified in mutant backgrounds containing receptor loss-of-function mutation, ethylene-insensitive mutations, and constitutive ethylene-response mutations. Ethylene-insensitive mutants of *ETR1* resulted in a post-transcriptional increase (2 to 3-fold) in levels of the mutant receptor. Treatment of seedlings with silver, which leads to ethylene-insensitivity, also resulted in a 2 to 3-fold increase in levels of ETR1 protein. Most other ethylene pathway mutations had relatively

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minor effects upon the expression of ETR1. Loss-of-function mutations in other members of the ethylene receptor family had little effect upon expression of ETR1, indicating that ETR1 does not functionally compensate for the loss of these receptors. My results indicate that mutations in *ETR1* can affect expression of the receptor at the post-transcriptional level. It is possible that the ethylene-insensitive forms of the receptor may have a slower rate of turnover than the wildtype receptors. The increase in levels of the mutant receptor may contribute to the dominant ethylene-insensitive phenotypes observed in the mutants, as signal output by the mutant receptor would be greater than that of the wildtype receptor due to the difference in protein levels. In addition, the mutant receptors may interact with wildtype receptors and convert them into ethylene-insensitive state via heteromeric interactions. A method as how this could occur has been proposed (Gamble et al., 2002). My results also clarify the mode of action of ethylene pathway mutations previously identified in *Arabidopsis* (Fig. 9). This study is described in chapter II.

The ethylene-insensitive mutant *etr1-1* and the constitutive ethylene response mutant *ctr1-2* can be used to mimic a maximal ethylene effect on plants without the interference of endogenous ethylene biosynthesis. Microarray technology was employed to analyze gene expression in these mutant plants under dark or light growth conditions. The gene expression in *ctr1-2* was compared to that in *etr1-1*, generating a data set of ethylene-regulated gene expression patterns. The effect of ethylene on gene expression displayed more cases of induction than of repression. Of all the 235 ethylene-regulated genes identified, 88% (207 genes) were induced and only 12% (28 genes) were repressed. The functional classification of the induced genes indicates that 45% (62 genes) of the

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induced genes were involved in signaling. Thirty-eight percent (10 genes) of the induced genes involved in signaling under both growth conditions are involved in ethylene signaling. The ethylene receptor genes *ETR2*, *ERS1*, and *ERS2* were significantly induced under both growth conditions. The ethylene receptor gene *ETR1* was induced to a limited extent, whereas *EIN4* did not show significant regulation by ethylene. The promoter regions of the *ETR2*, *ERS1*, and *ERS2* genes contained sequences with similarity to a known primary ethylene response element (PERE), suggesting that induction of these genes could be a primary response of the ethylene signal transduction pathway. The microarray result confirms that the expression of *ETR2*, *ERS2* and *ERS1* is up-regulated by ethylene at the transcriptional level, which had been previously demonstrated by northern blot analysis (Hua et al., 1998). These genes are potentially induced via a primary ethylene response element. These results suggest that ethylene can regulate the expression of its receptor genes potentially as a mechanism to re-sensitize plants to changes in ethylene levels (Fig.15). This work is described in chapter III.

Dynamic changes in the expression of ethylene receptors were observed in *Arabidopsis* in response to abiotic stress. The expression levels of *ETR1*, *ETR2*, and *ERS1* decreased significantly after exposure to salt stress, while *ERS2* and *EIN4* remained unchanged. Changes in gene expression were reflected at the protein level as levels of the ETR1 protein decreased following salt and osmotic stress, but with a significant lag. This lag could arise from a requirement for the ETR1 message to fall below a threshold level before protein levels are affected or be due to a slow rate of turnover for the ETR1 protein. Consistent with activation of ethylene pathway playing a role in salt and osmotic stress responses, I found that the constitutive ethylene response mutant *ctr1-2* exhibited

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more resistance to salt and osmotic stress than the ethylene-insensitive mutants *etr1-1* and *ein2*. As ethylene receptors are negative regulators of the ethylene signal transduction pathway, a decrease in receptors is predicted to increase the sensitivity of the plant to ethylene or to activate ethylene responses independent of ethylene. This study is described in chapter IV.

In conclusion, the intrinsic regulation of ethylene receptor expression has been studied in this dissertation (Fig. 23). Analyses revealed how plants modulate the levels of their ethylene receptors when a mutation occurs in the genes of the pathway or when plants encounter environmental stimuli, such as abiotic stress. Mutations in an ethylene receptor gene can affect its protein level in a posttranscriptional manner, and it is likely that the changes in expression level contribute to the mutant phenotype. The expression of ethylene receptor genes can also be up-regulated as demonstrated by comparing gene expression levels in reciprocal mutants of the ethylene pathway. The expression of ethylene receptors is also regulated by abiotic stress, in this case salt and osmotic stress. There are differences in the response of ethylene receptors to ethylene and to salt and osmotic stress. Ethylene, as well as salt and osmotic stress, regulated the expression of *ETR1*, *ETR2* and *ERS1*. The expression of *ERS2* was regulated by ethylene but not salt and osmotic stress. The expression of *EIN4* was not regulated by any of these factors. Based on sequence analysis, the ethylene-inducibility of *ETR2*, *ERS1* and *ERS2* is potentially controlled by a primary ethylene response element found in the promoter regions of each of these genes. Other promoter elements are likely to be involved in the transcriptional regulation of *ETR2*, *ERS1* and *ETR1* in response to salt and osmotic stress. The degree of response was different among different members of the receptor family.

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The expression of *ETR2* displayed the strongest and quickest changes in response to both ethylene and salt stress. In contrast, the expression of *ETR*1 showed a slower and less pronounced response to both stimuli. Differences in regulation may represent a careful fine-tuning of responses between members of the ethylene receptor family.

Changes in expression levels of the ethylene receptors provide a potential means by which ethylene signal transduction can be activated or repressed. My results lend insight into the regulation of ethylene receptor levels, and clarify potential mechanisms by which such regulation may occur. Understanding the regulation of ethylene signal transduction will not only enrich our knowledge about hormonal signaling in plants, but also provide a potential means to manipulate the ethylene response in plants of agronomic value.

Future Directions of this Research

A complete analysis of changes in ethylene receptor expression at the protein level has not been possible due to a lack of antibodies for other receptor members besides ETR1. In chapter III, the induction of *ETR2*, *ERS1*, and *ERS2* gene by ethylene was observed. In chapter IV, changes in transcript level were observed for the ethylene receptor genes *ETR2*, *ERS1*, and *ETR1*. However, analysis at the protein level was only possible for ETR1 because a highly specific antibody is only available for ETR1. Therefore preparation of highly specific antibodies for the remaining four members of the receptor family is very important to the study of regulation of ethylene receptors. With the availability of antibodies against all five ethylene receptor members, one could investigate how the protein levels of the five members change in response to various

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conditions such as introduction of mutations or transgenes, and environmental stimuli. For example, ethylene-insensitive mutations in *ETR2* and *EIN4* are already available. Do these mutations cause an increase in their receptor protein level as observed for ETR1? Is this a common feature shared by other receptor members or just for ETR1? These questions can be answered if antibodies against ETR2 or EIN4 are available.

In Chapter III, I proposed that a primary ethylene response element (PERE) may regulate ethylene induction of the *ETR2*, *ERS1*, and *ERS2* genes. This proposal awaits experimental demonstration. One approach would be to clone these putative regulatory elements along with a minimal promoter in front of a reporter such as GUS, and then examine the inducibility of GUS in transgenic *Arabidopsis*. If this putative promoter element confers ethylene inducibilily, the reporter gene will be induced in response to ethylene.

Based on the results from this research, the regulation of ethylene receptor levels potentially provides a means by which ethylene signal transduction can be activated and repressed without the presence of the hormone ethylene. Experiments should also be done to examine the effect of manipulation of the ethylene receptor levels. One way to manipulate the ethylene receptor level in plants is to over-express ethylene receptor genes in *Arabidopsis* (Appendix). Using transgenic plants, we will be able to investigate the effect of over-expressing ethylene receptor genes upon ethylene signal transduction and the sensitivity of plants to salt and osmotic stress (chapter IV). These two tests directly relate to the analyses that I have done in chapter II and IV, and will provide more information on regulation of ethylene responses by means of regulating ethylene receptor levels.

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Another way to manipulate the ethylene receptor level in plants is to eliminate ethylene receptor genes in plants. I already examined the effect of *ETR2*, *EIN4*, and *ERS2* loss-of-function mutations upon the expression of ETR1 (chapter II, figure 7). Loss-of-function mutations in four of the five receptors are available now. Double and triple mutations arising from combinations of these four single loss-of-function mutations are also available. We can examine the effect of these mutations upon a plant's sensitivity to salt and osmotic stress by examining the germination rate of these mutants in response to various osmoticums (chapter IV). With the information generated from over-expressing and loss-of-function plants, a better understanding of how ethylene responses are controlled through regulation of ethylene receptor levels can be obtained.

Arabidopsis is a model system to study plant biology (chapter I). The knowledge we obtain from *Arabidopsis* can be applied to agronomic plants, although *Arabidopsis* itself does not have agronomic value. The information about regulation of ethylene signal transduction in *Arabidopsis*, for example, provides a potential means to alter ethylene-response-associated processes such as seed germination, flower abscission, fruit-ripening and organ senescence in agronomic plants as desired. I believe in the future, agriculture will benefit from my studies on the regulation of ethylene signal transduction.

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APPENDIX

OVEREXPRESSING ETR1 AND *etr1-1* GENES WITH AN INDUCIBLE PROMOTER IN *ARABIDOPSIS*

Introduction

In the previous chapters, the regulation of endogenous ethylene receptor levels was examined. These analyses revealed how plants modulate the levels of their ethylene receptors when a mutation occurs in various elements of the ethylene pathway or when plants encounter salt and/or osmotic stress. Results from these analyses indicate that changes in ethylene receptor levels provide a means by which ethylene responses can be activated or repressed. Therefore, manipulating ethylene receptor levels in the plant could control ethylene responses. For example, increasing the sensitivity to ethylene in plants at the fruit-bearing stage could be used to stimulate the ripening of fruits. This could be accomplished by reducing the ethylene receptor levels in the plant. In contrast, decreasing the sensitivity to ethylene in ornamental plants could be used to delay the abscission of flowers and leaves. This could be accomplished by increasing the ethylene receptor levels in the plant.

Because ethylene regulates development and growth throughout the life of a plant, constitutive overexpression of ethylene receptor gene may cause problems in growth and development. For instance, seeds from the transgenic plants may not germinate effectively due to over-repression of ethylene responses arising from high expression level of the ethylene receptors. In addition, results form our lab suggest that

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such overexpression may not be experimentally feasible. A transgenic line overexpressing a fusion of *ETR1* and *GFP* using a strong constitutive promoter CaMV35S was generated. We observed that the endogenous ETR1 protein level was greatly reduced compared to the wildtype plant, and that the protein level of the transgenic ETR1-GFP fusion was similar to normal levels of ETR1. This result indicates that it is difficult to overexpress *ETR1* using a constitutive promoter. Possible reasons for this result include transcriptional gene silencing (Steimer et al., 2000), post-transcriptional gene silencing (Dalmay et al., 2000), and potential effects of overexpression on translation or turnover of the protein. Therefore, regulated expression may be crucial in obtaining high expression levels of the ethylene receptors.

Figure A. The T-DNA region of the glucocorticoid-inducible pTA7001 vector RB, right T-DNA border; 35S, CaMV 35S promoter; GVG, glucocorticoid-inducible transcription factor; E9, pea *rbc*S-E9 polyadenylation sequence; NOS, nopaline synthetase polyadenylation sequence; HPT, hygromycin phosphotransferase; NOS, nopaline synthetase terminator; 3A, pea *rbcS*-3A terminator; cloning sites SpeI and XhoI; 6xUASgal4, GVG-regulated promoter; LB, left T-DNA border.

Recently, a novel chemical induction system for inducible transcription in plants has been developed (Aoyama and Chua, 1997) (Fig.A). This induction system employs the regulatory mechanism of the vertebrate steroid hormone receptor, the glucocorticoid receptor (GR). GR is not only a receptor but also a transcription factor that activates transcription from promoters containing glucocorticoid response elements (GRE) in the presence of a glucocorticoid. The new induction system contains a chimeric transcription factor (encoded by the *GVG* gene). This *GVG* gene consists of the DNA-binding domain of the yeast transcription factor GAL4, the trans-activating domain of the herpes viral

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protein VP16, and the hormone-binding domain of the rat Glucocorticoid receptor. The GVG protein is made and retained in an inactivated state until binds to a glucocorticoid such as dexamethasone, which is a strong synthetic glucocorticoid that readily permeates the plant cell (Aoyama and Chua, 1997). Once bound to a glucocorticoid, GVG becomes activated and binds to the target promoter of six copies of the GAL4 upstream activating sequence (6xUAS$_{gal4}$). Transcription of the target gene is then initiated by the action of the VP16 trans-activating domain of GVG. By use of this inducible promoter system, the expression of foreign genes can potentially be regulated at any stage during the life of a plant and for any length of time.

Here, I examine the use of the glucocorticoid-inducible system to overexpress genes for *ETR1* and the ethylene-insensitive mutant *etr1-1* of *Arabidopsis*. These two transgenes were introduced into three different backgrounds: (1) wildtype, (2) the ethylene-insensitive mutant *ein2* (Alonso et al., 1999), and (3) the *sde1-1* mutant defective in post-transcriptional silencing (Dalmay et al., 2000). My results indicate that this induction system functions in *Arabidopsis* as elevated levels of ETR1 and etr1-1 protein were detected upon induction with dexamethasone.

Materials and Methods

Construct Design and Transformation of Plants

The genomic *ETR1* gene and the ethylene-insensitive mutant *etr1-1* gene were amplified by means of Polymerase Chain Reaction (PCR) from genomic clones (Chang et al., 1993). One set of oligonucleotide primers, 5'-GTCGACCATACCGGTTTAGAGTTTCT-3', 5'-GTCGACAGTAAGAACGAAGAAGAAGT-3', was used to amplify both genes starting at the first

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exon (556 bp upstream of the start codon), and ending 178 bp downstream of the stop codon. The 3,403 bp PCR products with *SalI* ends were cloned into the *XhoI* site of the glucocorticoid-inducible vector pTA7001 (Aoyama and Chua, 1997) (Fig. A).

Constructs in the pTA7001 vector were introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed by the dipping method (Bent and Clough, 1998) into *Arabidopsis* ecotype Columbia, the ethylene-insensitive mutant *ein2* (Alonso et al., 1999), and the *sde1-1* mutant defective in post-transcriptional silencing (Dalmay et al., 2000). Primary transgenic plants (T^0) were allowed to self-pollinate under standard growth conditions (22°C, 18 hr light) and seeds (T^1) were collected. Transgenic progeny were selected by plating seeds on half-strength of Murashige and Skoog medium supplemented with 50 μg/ml hygromycin. $Hygro^R$ seedlings were transferred to soil, and homozygous lines identified in subsequent generations based on segregation for hygromycin resistance.

Plant Material and Growth Conditions

Arabidopsis etiolated seedlings were grown as follows: seeds were surface-sterilized and sown on Petri dishes cantaining 1% (w/v) agar and half-strength Murashige & Skoog basal medium (pH5.65 - 5.85) with Gamborg's vitamins (MS media, Sigma). Seeds were stratified for 2 days at 4°C, then light-treated for 12 hr prior to growth at 22°C in the dark for another 3.5 days.

For plants grown in hydroponic growth medium: seeds were surface-sterilized and put in flask containing 25 ml of 2%(w/v) of sucrose and full-strength Murashige & Skoog basal medium (pH5.65 - 5.85) with Gamborg's vitamins (MS media, Sigma). The flask

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was kept shaking at 100 rpm at 22°C under continuous illumination (45 μE m^{-2} sec^{-1}) with cool-white fluorescent lights.

Glucocorticoid treatment

The glucocorticoid used was dexamethasone (DEX) (Sigma). A 30 mM stock solution was made by dissolving dexamethasone in ethanol. For plants grown on agar medium or in hydroponic growth medium , dexamethasone was supplemented to medium to a final concentration of 10 μM. The same volume of ethanol was added to control samples.

Protein Isolation and Immunoblot Analysis

For isolation of *Arabidopsis* membranes, plant materials were homogenized at 4°C in extraction buffer (30mM Tris-HCl, pH 8.5, 150mM NaCl, 10mM EDTA, 20% glycerol) containing 1mM phenylmethylsulfonyl fluoride, 1μg/mL pepstatin, 10μg/mL aprotinin, and 10μg/mL leupeptin as protease inhibitors. The homogenate was strained through Miracloth (Calbiochem) and centrifuged at 8,000 x g for 15min. The supernatant was centrifuged at 100,000 x g for 30min, and the membrane pellet then resuspended in resuspension buffer (10mM Tris, pH 7.5, 150mM NaCl, 1mM EDTA, 10% glycerol) with same protease inhibitors at used for homogenization. Protein concentration was determined by a modified version of the Lowry assay (Schaller and DeWitt, 1995). Bovine serum albumin was used as a standard.

For immunoblot analysis, membranes were mixed with SDS-PAGE loading buffer containing 100mM of dithiothreitol and incubated at 37°C for 1hr. Proteins were fractionated by SDS-PAGE using 8% (w/v) polyacrylamide gels. After electrophoresis, proteins were electrotransferred to PVDF Immobilon membrane (Millipore). Western

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blotting was performed by using anti-ETR1 (401-738), or anti-H^+- ATPase polyclonal antibodies. Anti-ETR1(401-738) was prepared against a GST-fusion protein with amino acids 401-738 of ETR1 (Schaller et al., 1995). The anti-H^+-ATPase antibody (DeWitt et al., 1996) was provided by Dr. M. Sussman (University of Wisconsin, Madison). Protein bands were visualized by enhanced chemiluminescence detection according to the manufacturer (Pierce). Densitometric analysis was performed using NIH Image (Version 1.60) after first scanning the exposed film and then capturing the image with Adobe Photoshop. Relative levels of the immunodecorated ETR1 were determined by comparison to a dilution series of ETR1.

Seedling Growth Response Assays

To examine the triple response of transgenic seedlings to ethylene (Chen and Bleecker, 1995; Hall et al., 1999), seeds were plated on Petri dishes containing 1% (w/v) agar and half-strength Murashige & Skoog basal medium (pH5.65 - 5.85) with Gamborg's vitamins (MS media, Sigma). Dexamethasone was included in the growth media to a final concentration of 10μM to induce expression of the transgenes. Plates were stratified at 4°C for 2 days, then placed at 22°C under fluorescent lights for 12 hr. Plates were then placed in 4-L sealed containers and seedlings grown in dark for 3.5 days. Ethylene (100 μL/L) was injected into sealed containers. Controls were grown in air.

Results

An inducible system for transgenic expression of *ETR1* and *etr1-1*

Two different constructs were made using the glucocorticoid-inducible vector pTA7001 (Aoyama and Chua, 1997), one containing a genomic *ETR1* gene, and the other

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a genomic *etr1-1* gene. Because ethylene receptors are negative regulators of the ethylene responses (Hua and Meyerowitz, 1998), the overexpression of the wildtype *ETR1* gene is predicted to reduce the plants' sensitivity to ethylene. The mutant *etr1-1* gene confers dominant ethylene insensitivity upon plants (Bleecker et al., 1988). Thus, upon induction of the *etr1-1* gene, dominant ethylene insensitivity should be conferred on the transgenic plants. Three different backgrounds were chosen for transformation: (1) wildtype (ecotype Columbia), (2) the ethylene-insensitive mutant *ein2* (Alonso et al., 1999), and (3) *sde1-1*, a mutant plant that has a defective post-transcriptional gene silencing mechanism (Dalmay et al., 2000).

The use of wildtype as a background will allow us to investigate the effect of overexpressing *ETR1* or *etr1-1* upon a plant with a normally functioning ethylene signal transduction pathway. As ethylene receptors are negative regulators of the ethylene responses (Hua and Meyerowitz, 1998), overexpression of the *ETR1* gene is predicted to result in decreased ethylene sensitivity in the transgenic plants. The decrease of ethylene sensitivity is potentially adjustable by varying the concentration of dexamethasone or the length of treatment.

ein2 is an ethylene-insensitive mutant resulting from a loss-of-function mutation in the downstream signaling element *EIN2*. Initial results indicated that loss-of-function mutations in *EIN2* resulted in complete ethylene insensitivity (Roman et al., 1995; Chen and Bleecker, 1995). However, in a recent analysis on ACC-induced root hair formation, it was found that the *ein2* mutant could still develop root hairs in response to ACC. This result suggests that *ein2* is not completely insensitive to ethylene (Cho and Cosgrove, 2001). We are interested in determining whether the introduction of the *etr1-1* gene into

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the *ein2* mutant background will result in an additive ethylene insensitivity with *ein2*. A stronger ethylene-insensitive phenotype could be identified if root hairs do not form when the transgenic plant is treated with ACC.

sde1-1 is a mutant defective in post-transcriptional gene silencing. Post-transcriptional gene silencing is a defense mechanism in plants that is similar to quelling in fungi and RNA interference in animals (Dalmay et al., 2000). A transgene that is similar to an endogenous gene can sometimes induce post-transcriptional gene silencing or cosuppression of the endogenous gene; this arises due to degradation of the mRNA of the endogenous gene. At the same time, the mRNA of the foreign gene is also degraded due to the same mechanism. The use of inducible promoter could avoid transcriptional gene silencing due to methylation of the promoter of silenced genes (Steimer et al., 2000). However, an inducible promoter would not avoid post-transcriptional gene silencing. Potentially, the *sde1-1* mutant will allow for overexpression of transgenic *ETR1* or *etr1-1* without post-transcriptional gene silencing.

Transgenes were successfully introduced into all three backgrounds (Table A). Homozygous lines have been obtained for: (1) transgenic ETR1 with wildtype background, (2) transgenic *etr1-1* with wildtype background, (3) transgenic ETR1 with *ein2* background, and (4) transgenic *etr1-1* with *ein2* background. Transgenic plants in the *sde1-1* mutant background have not been screened for homozygous lines. The number of independent transgenic lines and corresponding homozygous lines obtained with each background is shown as in Table A.

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Table A. Transgenic lines obtained

Transgene	Background	Hygro^R Lines	Homozygous Lines
ETR1	wildtype	27	3*(8BA,8BB,8BF.8CB,8CF,10AB,10AC,10AD)
etr1-1	wildtype	35	3 (1AB,1AC,2BB,2BD,4AD,4AE, 5AA,5AE)
ETR1	*ein2*	28	5 (1CE,4BA,4BB,6CA,6CB,7AC,10AA,10AD)
etr1-1	*ein2*	37	3* (3AA,3AB,3AD,3GD,3AE)
ETR1	*sde1-1*	16	N/A
etr1-1	*sde1-1*	12	N/A

* : Screen is incomplete. There are more potential lines to be screened.
N/A : not available.

Induction of ETR1 and etr1-1 protein by treatment with dexamethasone

The inducibility of ETR1 and etr1-1 in transgenic wildtype plants was examined by immunoblot analysis using an antibody against ETR1 (Fig. B). The transgenic lines used for immunoblot analysis were segregating T^2 plants. The lines gETR1 (2B), gETR1 (3C), getr1-1 (2B), getr1-1 (3A), and getr1-1 (1A) all contain a single T-DNA insertion based on analysis of hygromycin resistance. Plants would thus be a segregating population of homozygous, heterozygous and wildtype plants (1:2:1). Line gETR1 (3B) has multiple T-DNA insertions. A transgenic wildtype line transformed with the pTA7001 vector itself was used as a control (pTA). All plants were grown in aqueous media for 3 weeks then treated with 10 μM DEX for 24 hr. Two out of the three transgenic lines transformed with the *ETR1* gene (gETR1 (3C) and gETR1 (3B)) showed a pronounced increase in ETR1 protein level when treated with DEX (Fig. B). One out of the three transgenic lines transformed with the *etr1-1* gene (getr1-1 (3A)) showed a small increase in the etr1 protein level when treated with DEX (Fig. B). DEX had no significant effect upon expression of ETR1 in the control line transformed with the pTA7001 vector.

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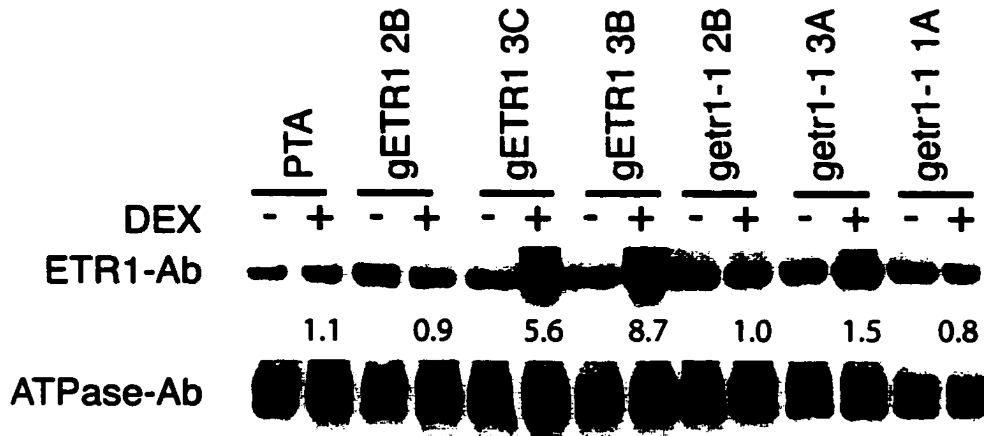

Figure B. Induction of ETR1 and etr1 expression in transgenic plants with wildtype background. Membranes were isolated from 3-week-old green plants grown in aqueous MS medium. PTA is a transgenic wild-type line transformed with the dex-inducible vector. Each lane was loaded with 10 ug of total protein. The immunoblot was probed with antibodies against ETR1 and H^{+}-ATPase as an internal control. Expression levels were quantified densitometrically and compared to untreated control (designated as 1).

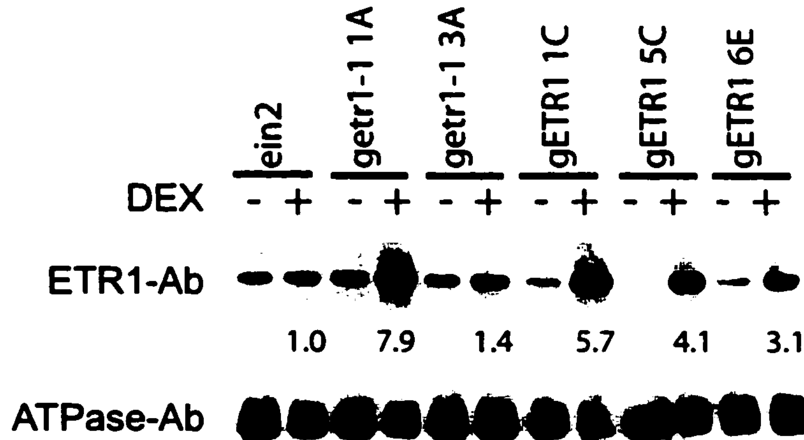

Figure C. Induction of ETR1 and etr1 expression in transgenic plants with the *ein2* background. Membranes were isolated from 4-day-old etiolated seedlings grown on agar and MS medium. Each lane was loaded with 10 ug of total protein. The immunoblot was probed with antibodies against ETR1 and H^{+}-ATPase as an internal control. Expression levels were quantified densitometrically and compared to untreated control (designated as 1).

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Transgenic T^2 lines in the *ein2* background were also examined for inducibility of ETR1 and etr1-1 using immunoblot analysis. The lines gETR1 (1C), gETR1 (5C), and getr1-1 (3A) all contain a single T-DNA insertion based on hygromycin resistance. The lines gETR1 (6E) and getr1-1 (1A) have multiple T-DNA insertions. The *ein2* mutant was used as a control. All plants were grown in the dark for 3.5 days on agar containing MS medium supplemented with 10 μM DEX. Lines transformed with either the *ETR1* or the *etr1-1* gene all displayed a pronounced increase in the ETR1 protein level when treated with DEX, except for the line getr1-1 (3A), whose induction level was relatively low (Fig. C). No increase in ETR1 protein level was observed for the *ein2* mutant treated with DEX.

Phenotypic study of the transgenic lines

To determine the effect of transgene expression on the transgenic plants, homozygous lines were examined for their ethylene responses. In presence of ethylene, wildtype seedlings grown in the dark display a "triple response", which is characterized by an inhibition of hypocotyls and root elongation, an exaggerated apical hook, and a thickening of the hypocotyls (Guzmán and Ecker, 1990). I performed a triple response assay to examine whether transgene expression causes any changes in the triple response, especially the hypocotyl length, of the transgenic plants.

Three independent wildtype lines and their sibling lines (8BA, 8CB, 10AB,10AC) transformed with the *ETR1* gene were examined. These lines had a wildtype phenotype. In contrast, one wildtype line transformed with *ETR1* (8CF) displayed a strong insensitivity to ethylene when grown on DEX-supplemented medium. To determine whether this ethylene insensitivity is caused by the transgene, other lines with the same

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parent plant as 8CF were also examined (8CA, 8CB, 8CD, 8CE). However, this insensitivity was not observed in any of the sibling lines. Therefore this insensitivity is not likely to be the result of overexpressing *ETR1* itself.

Four wildtype lines transformed with genomic *etr1-1* gene (1AA, 2BC, 3AA, 4AB) showed strong ethylene-insensitivity when grown on DEX-supplemented medium. However, this insensitivity was also observed when the transgenic plants were grown on medium without DEX, indicating that there was a basal level of *etr1-1* being induced even without DEX.

Five transgenic lines in *ein2* background were examined (gETR1 (1C), gETR1 (5C), gETR1 (6E), getr1-1 (1A), getr1-1 (3A)), and all were insensitive to ethylene as expected. Seeds of these lines will be sent to the Cosgrove lab at Pennsylvania State University to determine whether the *etr1-1* transgene affects ACC-induced root hair formation.

It should be noted that the use of the antibiotic hygromycin for selection is not ideal. Even the growth of resistant transformants was severely impaired. The seedlings were not very healthy and frequently died after transplanted to soil. This effect was particularly severe with the T^2 transgenic *sde1-1* plants. When the T^2 seeds were plated on hygromycin plates to screen for homozygous lines, 50% of the seeds did not germinate.

Another observation is that the application of DEX to growth media can result in an abnormal growth of seedlings, including wildtype seedlings. The seedlings were stunted, and the hypocotyls frequently coiled when grown on vertically oriented plates.

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Discussion

The use of the inducible promoter to overexpress the *ETR1* gene is effective. Results from immunoblot analysis indicate that a substantial increase in the ETR1 protein level can be obtained after inducing with DEX (Fig. B, C). In transgenic wildtype plants, a 5.6- and 8.7-fold increase in ETR1 protein level was observed for lines gETR1 (3C) and gETR1 (3B) respectively. In transgenic ein2 plants, a 7.9- and 5.7-fold increase in ETR1 protein level was observed for lines getr1-1 (1A) and gETR1 (1C) respectively. As previously described, using a 35S promoter to express the *ETR1* gene did not result in an elevated ETR1 protein level. Using the *ETR1* genomic promoter to express the *ETR1* gene resulted in only a 4-fold increase in ETR1 protein level (Chen et al., 2002). Thus use of the inducible promoter allows for greater overexpression of the *ETR1* gene than has been found with any other methods that we have tested. However, the induction system has a certain level of leakiness. This leakiness was observed as wildtype plants transformed with the *etr1-1* gene display ethylene insensitivity even without the addition of DEX.

Two different methods were used for analysis of induction: (1) addition of DEX to 3-week-old mature plants grown in aqueous media, and (2) addition of DEX to etiolated seedlings grown on agar. In both cases, increased ETR1 protein level was observed. The increase in protein level was more pronounced in etiolated seedlings. However, this may be because the etiolated seedlings were grown in the presence of DEX for 3.5 days while the mature plants were just treated with DEX for 24 hours. The potential advantage of using plants grown in aqueous media is that the induction can be controlled easily by varying the length of treatment.

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Although I already obtained several homozygous lines and performed an initial analysis of these lines, this project awaits additional experimentation. The remaining work includes: (1) complete the screening for homozygous lines, particularly in the *sde1-1* background, (2) perform an ethylene dose-response assay to examine whether wildtype seedlings transformed with the *ETR1* gene display a change in sensitivity to ethylene; (3) examine the ETR1 and etr1 protein levels in the transgenic *sde1-1* mutant upon DEX-induction to see if the increase in protein level is higher than that observed with transgenic wildtype or *ein2* plants; (4) examine the sensitivity of transgenic plants transformed with the *ETR1* gene to salt and osmotic stress, as overexpression of *ETR1* is predicted to increase the sensitivity to salt and osmotic stress in plants (see chapter IV); (5) collaborate with the Cosgrove's laboratory to determine whether overexpression of *etr1-1* confers greater ethylene-insensitivity upon *ein2* plants.

Although further characterization is needed, the lines I already obtained are potentially useful for other experiments. Some of the future experiments such as (2), (4) and (5) can directly take advantage of the lines I already obtained. In addition, several lines that overexpress the *ETR1* gene are being used by my lab colleague Yi-Feng Chen for purification and analysis of the ETR1 receptor complex.

In conclusion, the glucocorticoid-inducible promoter system can be used to overexpress the ethylene receptor genes *ETR1* and *etr1-1*. However, this system still has some potential problems. First, this system has leakiness, as my phenotypic study indicates that the transgene may be induced to some extent in plants without the application of the inducer glucocorticoid. Second, selection with the antibiotic

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hygromyocin is not ideal, as it limits the viability of transformants. With some improvements, this system will be a powerful tool to study gene expression in plants.

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