THE ETHYLENE RECEPTOR MULTIGENE FAMILY: INSIGHTS ON EXPRESSION, LOCALIZATION AND FUNCTION IN ARABIDOPSIS AND TOMATO

By

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This work is dedicated to the few who do not try to fit their data to existing models, but instead try to understand the meaning of their findings despite them.

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THE ETHYLENE RECEPTOR MULTIGENE FAMILY: INSIGHTS ON EXPRESSION, LOCALIZATION AND FUNCTION IN ARABIDOPSIS AND TOMATO

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Chair: Harry J. Klee
Major Department: Plant Molecular and Cellular Biology

Ethylene is a plant hormone that affects several aspects of growth and development. Ethylene receptors comprise a diverged multigene family, with six members in tomato and five in Arabidopsis. The expression patterns of these receptors and their localization in the cell were investigated in this work, as a means to understand ethylene signal transduction. I show here that the tomato receptors have the same gene structure as the Arabidopsis receptors, and they localize to the endoplasmic reticulum. I also investigated the expression pattern of one of the tomato receptors, *NEVERRIPE*, which is regulated throughout development. I show here that ethylene itself can regulate its receptor levels, and my studies in Arabidopsis take advantage of the large mutant collection available for this model species.

The ethylene receptors show sequence similarity to bacterial two-component histidine kinases. These receptors can be divided into two subfamilies, 1 and 2. It has been previously shown that a subfamily 1 Arabidopsis ethylene receptor, ETR1,

autophosphorylates *in vitro* on a conserved histidine residue. However, sequence comparisons between the five ethylene receptor family members suggest that subfamily 2 members do not have the motifs necessary for histidine kinase activity. Here I show that all five Arabidopsis ethylene receptor proteins autophosphorylate *in vitro*. I analyzed the nature of the phosphorylated amino acids by acid/base stability and two-dimensional thin layer electrophoresis, and demonstrated that unlike ETR1 all other ethylene receptors autophosphorylate predominantly on serine residues.

ERS1, the only other subfamily 1 receptor in Arabidopsis, is able to phosphorylate on both histidine and serine residues in the presence of Mn^{2+}. However, this activity is lost when ERS1 is assayed in the presence of both Mg^{2+} and Mn^{2+}, suggesting that histidine autophosphorylation may not occur *in vivo*. Furthermore, mutation of the histidine residue conserved in two-component systems does not abolish serine autophosphorylation, discarding the possibility of a histidine to serine phosphotransfer. My biochemical observations complement the recently published genetic data that histidine kinase activity is not necessary for ethylene receptor function in plants and suggests that ethylene signal transduction does not occur through a phosphorelay mechanism.

CHAPTER 1
INTRODUCTION

There are several well characterized hormones in plants, generally referred to as *phytohormones*, whose roles and modes of action are diverse. Phytohormones do not follow the "classical" definition of animal hormones of molecules synthesized at a specific site that are transported to their site of action, where changes in their concentration lead to physiological responses (reviewed in Davies, 1995). The most general definition for a phytohormone is a compound made by the plant that affects physiology at concentrations lower than those of nutrients and vitamins. When concentrations of these compounds are altered, development is affected (reviewed in Gaspar et al., 2003). The plant responses to hormones are mostly through growth and development such as: tissue regeneration following herbivore feeding, redirection of growth following shading or changes in light sources, and desiccation of the seed following its maturation. Hormonal responses, however, are rarely due to a single phytohormone, as hormone networks are very prominent in plants. Most physiological responses are due to multiple signals, and the same signals can also lead to a variety of responses depending on how they are combined (reviewed in Ross and O'Neill, 2001; Weyers and Paterson, 2001).

Phytohormones can be produced from a variety of molecules including amino acids, nucleotides, terpenoids, fatty acids and carotenoids. Most phytohormones do not have a specific site of synthesis; they can be synthesized by different tissues and even by

different cells of those tissues. Plant physiology is controlled by these chemical messages, which can be transported throughout the plant, but they can also act on the same tissue and even the same cell in which they are synthesized. Some phytohormones require transport to their site of action, where they mediate responses in a dose-dependent manner. The lack of a continuous circulation system in plants seems to aid in the directional flow of these compounds (reviewed in Davies, 1995; Weyers and Paterson, 2001).

Phytohormones are thought to signal through their interaction with specific receptor molecules. These receptors can be at the cell surface or inside the cell; they activate signal transduction pathways that induce or inhibit gene expression and cellular functions, including changes in membrane potential. Most studies so far have focused on transcriptional regulation by hormones. Several promoter regions have been identified that contain *cis*-acting elements that confer hormone responsiveness. These elements recruit specific transcription factors that assemble at the promoter, forming complexes that can induce or repress transcription (Gaspar et al., 2003).

There are several aspects to a hormone response, allowing for multiple points of regulation. For a given phytohormone, plants can regulate synthesis, transport, uptake and metabolism of the active molecule, as well as perception and cellular response. These steps can be developmentally regulated, which may involve regulation by other hormones. Hormone concentration is controlled by metabolism, at the levels of biosynthesis, degradation and/or inactivation. Phytohormones can be inactivated by conjugation to sugars or amino acids. Some of these conjugations can also be a form of storage as some conjugations are reversible (Kende and Zeevaart, 1997). Plants can

regulate changes in hormone concentration, but the "sensitivity" of the tissue to the hormone also changes throughout development. A change in tissue sensitivity is defined as the capacity of a tissue to vary its ability to respond to a given concentration of a stimulus (i.e., hormone) throughout development. It is not clear what regulates changes in tissue sensitivity; it could be due to changes in receptor concentrations, changes in the affinity between the receptor and the hormone, changes in signal transduction components, or combinations thereof (reviewed in Weyers and Paterson, 2001; Gaspar et al., 2003).

Ethylene (ethene; C_2H_4) is a simple gaseous molecule and one of the best understood plant hormones. It is responsible for coordinating several aspects of plant physiology and, even though it has been studied for over a century, there is still very little understanding as to how the plant interprets the ethylene signal. The purpose of this work was to increase our understanding of the mechanism of ethylene signaling through the analysis of the ethylene receptors.

Ethylene in Plant Biology

Depending on the species, cultivar, tissue, and stage of development, ethylene may signal a variety of biological responses. Some of the roles of ethylene are to coordinate leaf and flower senescence, defense responses, fruit ripening, leaf and fruit abscission, and seed germination (reviewed in Abeles et al., 1992). Flower development is regulated by ethylene and pollination elicits a burst in ethylene production in the style and stigma of the flower that leads to petal senescence. This process is very dramatic in orchid flowers as they last over 80 days if unpollinated, but senesce 2 days after pollination or ethylene treatment (O'Neill et al., 1993; Zhang and O'Neill, 1993). Changes in ethylene synthesis are observed through development but also in response to environmental

changes. Biotic and abiotic stresses can lead to increases in ethylene concentration, and depending on the pathogen, ethylene can induce defense responses or suppress them. Suppression of plant defense responses is used to exacerbate disease symptoms and to kill the tissue in order to control pathogen spread (reviewed in Abeles et al., 1992; Bleecker and Kende, 2000).

The agriculture industry has sought control of fruit ripening as a way of preventing fruit from spoiling before it reaches the consumer, and ethylene biosynthesis and perception has been a target for breeding and genetic manipulations. The fruit ripening process is complex and includes loss of chlorophyll, production of pigments, production of volatiles involved in flavor and aroma, softening of the flesh of the fruit, and abscission of the fruit. Some classes of fruits, called *climacteric*, show a steep increase in ethylene production at the mature green stage that is accompanied by rise in cellular respiration. Examples of climacteric fruit are apple, banana, cantaloupe and tomato (reviewed in Mattoo and Suttle, 1991). Tomato fruit ripening involves changes in ethylene production and gene expression at the mature green stage, before the onset of ripening. Some genes change their expression pattern due to changes in ethylene levels while others are developmentally regulated (Lincoln et al., 1987; Lincoln and Fischer, 1988). Ripening starts at one region and quickly spreads throughout the rest of the fruit. Ethylene diffuses freely from cell to cell because it is liposoluble. It also stimulates its own biosynthesis, which helps amplify the process in a positive feedback loop (reviewed in Bleecker and Kende, 2000).

In most plants, sensitivity to ethylene changes with development. Ethylene applied to immature green fruit will not induce ripening, but after ripening has initiated ethylene

speeds the ripening process. However, the plant is able to perceive ethylene at an early stage and remembers the amount of ethylene to which it has been exposed during development. Gassing an immature green fruit with ethylene will not induce ripening but the number of days until ripening starts is reduced (reviewed in Abeles et al., 1992). It is not clear what determines the sensitivity of the tissue to ethylene. One possibility is that the developmental regulation of ethylene receptor expression would determine levels of ethylene perception. It has been suggested that one of the tomato receptors, NEVERRIPE (NR), might be rate-limiting before fruit ripening, as its messenger RNA accumulates concomitant with the increases in ethylene production during ripening (Lashbrook et al., 1998). It has also been shown that changes in receptor concentration in tomato, by antisense or over-expression, clearly affect the plant's sensitivity to ethylene (Ciardi et al., 2000; Tieman et al., 2000). An auto-inhibitory effect of ethylene on its own synthesis is observed in immature tomato fruit, which is then converted to an inducible effect at the onset of ripening. This difference in effect might be related to differential expression and regulation of ethylene biosynthetic enzymes by ethylene (Atta-Aly et al., 2000). However, sensitivity to ethylene has also been shown to be independent of ethylene production in orchids. For example, inhibition of ethylene biosynthesis by aminooxy acetic acid has no effect on the plant's sensitivity to the hormone (Porat et al., 1994).

Another process controlled by ethylene is stem and petiole growth. When plants are grown in the dark they normally express an etiolated phenotype, which consists of a pale color and a tall and slim elongation of the stem. In the presence of ethylene, plants show a phenotype termed the *triple response* rather than the etiolated phenotype. This

response is characterized by inhibition of shoot elongation accompanied by radial stem expansion, apical hook tightening, and loss of gravitropism. Ethylene is induced upon germination and the triple response morphology helps protect the seedling's apex and young leaves during soil emergence. Paradoxically, ethylene inhibits stem elongation in terrestrial plants but promotes rapid growth of stems in semi-aquatic plants, such as rice. Ethylene accumulates in submerged tissues, as it cannot diffuse as well through water as in air, and the rapid stem elongation leads to foliage formation above water (reviewed in Mattoo and Suttle, 1991; Bleecker and Kende, 2000).

The triple response is an ethylene phenotype that has been very useful for genetic studies and this seedling phenotype has been exploited in order to search for mutants that show deviant behavior in the presence or absence of ethylene (Guzman and Ecker, 1990). These mutant screens yielded two classes of mutants: those that were insensitive to the presence of ethylene and do not show the triple response, and those that show a constitutive triple response in the absence of ethylene (reviewed in Kieber and Ecker, 1993; Kende and Zeevaart, 1997). These mutants have helped elucidate the ethylene signal transduction pathway and suggest that there are two levels of regulation of the plant's response to ethylene: one at the level of biosynthesis and one at the level of perception.

Ethylene Biosynthesis in Plants

Ethylene is synthesized from carbons C-3 and C-4 of methionine via two intermediates: S-adenosyl-L-methionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC), as seen in Figure 1-1. ACC synthase (ACS) and ACC oxidase (ACO) are the major enzymes in ethylene biosynthesis, where ACS catalyzes the first committed step in ethylene synthesis and is the primary step regulating ethylene production. The

conversion of SAM to ACC by ACS releases 5'-methylthioadenosine (MTA). MTA is recycled to methionine by the Yang cycle, at the expense of ATP. This permits a continuous production of ethylene without depleting the plant's methionine pool (Bleecker and Kende, 2000; Wang et al., 2002). ACC can be converted to ethylene by ACO or it can be inactivated by conjugation to produce malonyl-ACC or glutamyl-ACC. This conjugate is a sink for ACC and reduces ethylene production (Alonso and Ecker, 2001). ACC oxidation produces CO_2 and cyanide as byproducts, the latter of which is converted to β-cyanoalanine to prevent the accumulation of toxic levels of cyanide (Wang et al., 2002).

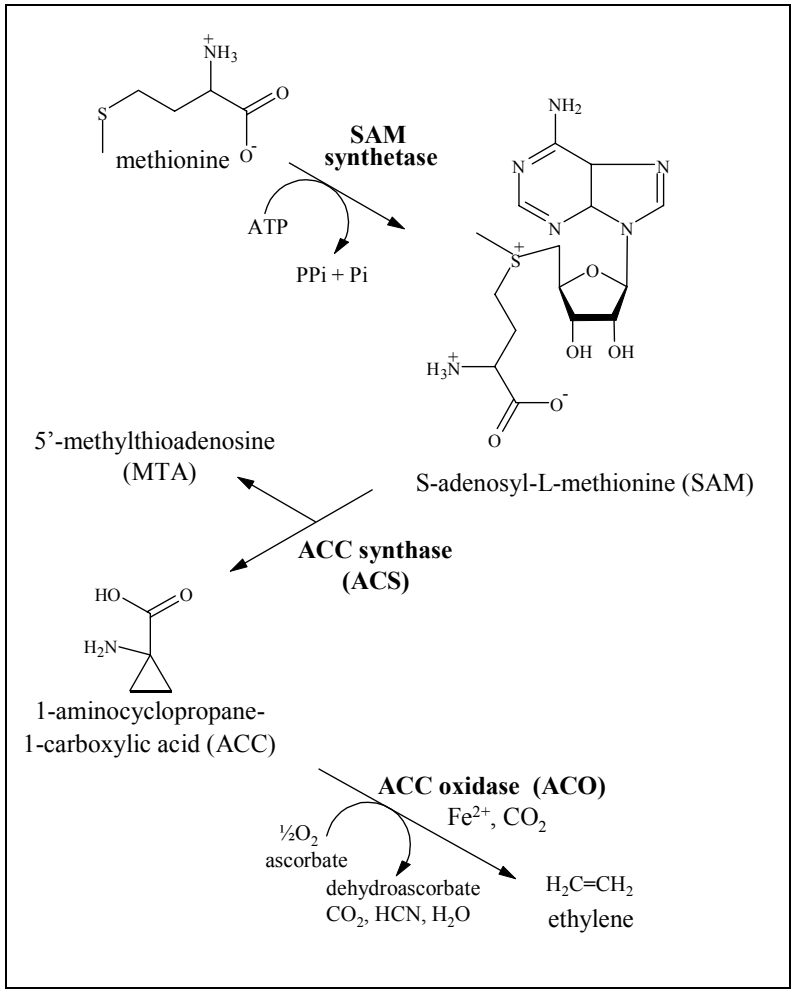

Figure 1-1. Ethylene biosynthesis pathway in plants.

Both ACS and ACO are encoded by multigene families, with 12 and 2 members in Arabidopsis, respectively. Transcripts of isoforms of these enzymes are differentially regulated during stages of plant development and in response to various environmental stimuli, including exposure to ethylene and other hormones (Kende and Zeevaart, 1997; Alonso and Ecker, 2001; Wang et al., 2002). Regulation of ACS occurs not only at a transcriptional but at a post-translational level. Mutations at the carboxyl-terminus of ACS leads to loss of protein turnover and increased ethylene synthesis (Vogel et al., 1998; Wang et al., 2002; Chae et al., 2003). It has also been suggested that the ETHYLENE OVERPRODUCING1 (ETO1) protein binds to the carboxyl-terminus of ACS and acts as an inhibitor of its activity by inducing protein turnover (Cosgrove et al., 2000; Alonso and Ecker, 2001; Wang et al., 2002). The binding of ETO1 to ACS might be regulated by phosphorylation of the carboxyl-terminus by a calcium-dependent protein kinase (Tatsuki and Mori, 2001). ACS is also subject to suicide inactivation, where the conversion of SAM to ACC leads to enzyme inactivation and degradation, which is also consistent with its high turnover rate (Satoh and Esashi, 1986; Satoh et al., 1993).

Ethylene Signal Transduction

Several components of the ethylene signal transduction pathway have been identified in the last two decades. Five proteins have now been identified in Arabidopsis as receptors for the plant hormone ethylene: ETHYLENE-RESISTANT1 (ETR1) (Chang et al., 1993), ETHYLENE RESPONSE SENSOR1 (ERS1) (Hua et al., 1995), ETR2 (Sakai et al., 1998), ETHYLENE INSENSITIVE4 (EIN4), and ERS2 (Hua et al., 1998). The ethylene signal is transmitted from these receptors to transcription factors, such as EIN3 (Chao et al., 1997) and other EIN3-LIKE proteins (EILs) (Solano et al., 1998), via a common pathway that includes CONSTITUTIVE-TRIPLE-RESPONSE1 (CTR1)

(Kieber et al., 1993), a MAP kinase cascade (Ouaked et al., 2003) and EIN2 (Chen and Bleecker, 1995). A putative CTR1-independent signaling pathway has also been proposed (Hua and Meyerowitz, 1998), but no components have yet been identified.

The most likely model for ethylene signaling is based on genetic data (Hua and Meyerowitz, 1998) and indicates that the ethylene receptors are in an "ON" state in the absence of ethylene, actively repressing downstream members of the pathway. Binding of ethylene switches the receptors to their "OFF" state, which releases repression of the ethylene signal transduction pathway (reviewed in Bleecker and Kende, 2000). Several dominant insensitive alleles of the receptors have been identified that impair ethylene binding and prevent release of the repressed state of the ethylene signal transduction pathway (Bleecker et al., 1988; Hua et al., 1995; Hua et al., 1998; Sakai et al., 1998). However, the mechanism of receptor function is not understood, because of a lack of biochemical data. Thus, "ON" and OFF" are used here in general terms. The proposed pathway for ethylene signal transduction is shown in Figure 1-2.

ETR1 was the first of five ethylene receptors identified in Arabidopsis and remains the best characterized receptor. ETR1 was identified due to a mutation that leads to lack of a triple response in the presence of ethylene. The *etr1-1* mutation confers a dominant ethylene insensitive phenotype and these plants have larger rosette leaves than wild-type. There is no significant change in ethylene synthesis but these plants show a 20% reduction in their ability to bind ethylene, when compared to wild-type plants (Bleecker et al., 1988). The amino-terminal region of ETR1 shows no similarities to known proteins and contains the ethylene binding-site (Schaller and Bleecker, 1995), while the

carboxyl-terminal region shows sequence similarity to bacterial two-component systems (Chang et al., 1993)

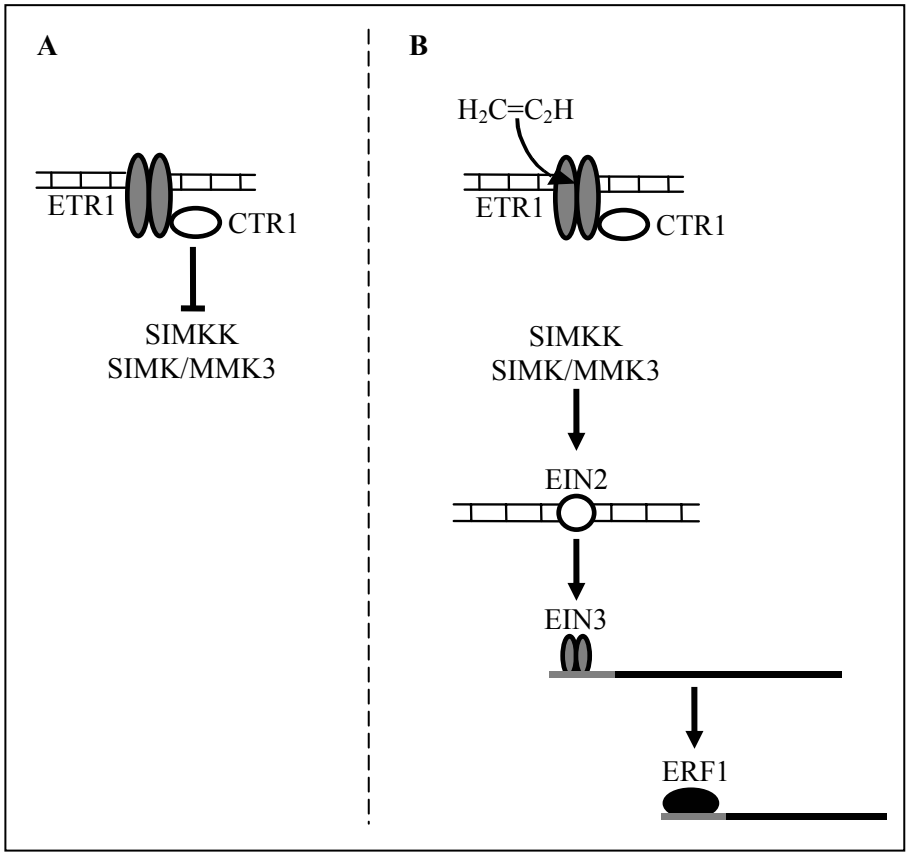

Figure 1-2. Proposed ethylene signal transduction pathway. A, receptors in "ON" state represses ethylene signal transduction. B, ethylene binding turns receptors "OFF", which leads to the activation of transcription factors.

In bacteria, two-component signal transduction systems are involved in a variety of responses, including osmotic regulation, chemotaxis, host recognition by pathogens, responses to changes in phosphate and nitrogen levels, and stress responses (reviewed in Stock et al., 2000). Traditional two-component signal transduction systems involve a sensor protein and a response regulator protein. In most cases, the sensor consists of a variable amino-terminal domain located in the periplasm, two trans-membrane regions, and a histidine kinase domain at the carboxyl-terminus. The histidine kinase domain

autophosphorylates in response to a given stimulus. The response regulator comprises a receiver domain and an effector domain. Following phosphorylation, the response regulator catalyzes the transfer of the phosphoryl group from a histidine residue in the sensor's kinase domain to an aspartate residue in its own receiver domain. The phosphorylation of the response regulator usually leads to a gain of DNA binding activity of its effector domain. Two-component proteins are also involved in more complex signaling pathways, termed *phosphorelays*. In these pathways the receptors are often hybrid proteins, containing a receiver domain at the carboxyl-terminus of their kinase domain. After autophosphorylation of the histidine residue in the kinase domain, the phosphoryl group is transferred intra-molecularly to the receiver domain. This phosphoryl group is subsequently transferred to a histidine-containing phosphotransfer protein (HPt), and then to a response regulator protein, completing a phosphorelay (reviewed in Stock et al., 2000).

ETR1 was localized to the endoplasmic reticulum (ER) (Chen et al., 2002) and receptor signaling seems to regulate the a MAP kinase cascade (Ouaked et al., 2003). Mitogen activated protein kinases (MAPKs) are enzymes that are activated following phosphorylation by a kinase named MAPK-kinase (MAPKK). If the latter is also activated by phosphorylation, the kinase that phosphorylates it is called MAPKK-kinase (MAPKKK). This succession of kinase activations is termed a MAP kinase cascade (reviewed in Alberts, 2002). CTR1 is a serine/threonine protein kinase with sequence similarity to the RAF family of protein kinases (Kieber et al., 1993). CTR1 is a MAPKKK and its kinase activity is necessary for repression of ethylene signaling (Huang et al., 2003). Mutations in the CTR1 gene are recessive and induce the triple response in

the absence of ethylene. This suggests that CTR1 acts as a repressor of ethylene response and the loss of this repression leads to a constitutive ethylene response (Kieber et al., 1993). Epistasis analysis suggests that CTR1 acts downstream of ETR1 (Roman et al., 1995) and it has been shown to interact with ETR1 *in vitro* and *in vivo* (Clark et al., 1998; Gao et al., 2003; Huang et al., 2003). Moreover, CTR1 localization to the ER is dependent on its interaction with ETR1 (Gao et al., 2003). Biochemical data suggest that SIMKK is a MAPKK involved in ethylene signaling. SIMKK seems to phosphorylate two MAPKs, SIMK and MMK3, which also seem to be involved in ethylene signal transduction (Ouaked et al., 2003).

MAP kinase cascades are eukaryotic signaling systems, while two-component regulators are prokaryotic systems. This integration of prokaryotic and eukaryotic mechanisms, however, is not unique to ethylene signaling. A similar system occurs in the yeast *S. cerevisiae*, for example, where there is only one histidine kinase sensor protein, the osmolarity receptor SLN1 (Ota and Varshavsky, 1993). SLN1 is a hybrid histidine kinase that transfers its phosphoryl group to a HPt, YPD1, which then transfers the phosphoryl group to the response regulator SSK1 (Maeda et al., 1994; Maeda et al., 1995). High osmolarity induces the deactivation of SNL1, which leads to the dephosphorylation of SSK1. This activates a MAP kinase cascade, where SSK2 is a MAPKKK, PBS2 is a MAPKK, and HOG1 is a MAPK (Posas and Saito, 1998). Five HPt homologues have been identified in Arabidopsis (reviewed in Hutchison and Kieber, 2002) and some have been shown to interact with ETR1 in yeast two-hybrid assay (Urao et al., 2000). There are also 22 response regulators in Arabidopsis (reviewed in

Hutchison and Kieber, 2002), some of which could be involved in ethylene signaling (D'Agostino et al., 2000).

Little is known about how the MAP kinase cascade regulates nuclear events in ethylene signaling. Epistasis analysis has positioned EIN2 downstream of the CTR1 (Roman et al., 1995). The EIN2 sequence predicts twelve trans-membrane helix domains at the amino-terminal domain. This region shows 21% identity to Nramp-related proteins, which are ubiquitous and seem to function as cation transporters (Alonso et al., 1999). The *ein2* loss-of-function mutants are recessive and completely insensitive to ethylene (Roman et al., 1995). The function of EIN2 in ethylene response is not clear, as no transport activity has been shown for this protein. One possibility is that it could be a transporter for a second messenger. However, EIN2 could also be a transporter for an important cofactor of one of the signal transduction components and not necessarily be directly involved in the pathway. Such is the case for the RESPONSIVE TO ANTAGONIST1 (RAN1) protein, which is a Menkes/Wilson disease-related copper transporter. As the receptors require copper as a cofactor, *ran1* loss-of-function mutants prevent the receptors from assembling properly (Hirayama et al., 1999).

Ethylene signal transduction regulates nuclear transcription factors, which coordinate ethylene response genes. EIN3 is a nuclear-localized protein with a novel DNA binding domain, containing coil and helical basic regions (Chao et al., 1997). EIN3 is a member of a multi-gene family of transcription factors, including *EIL1*, *EIL2*, and *EIL3* (Solano et al., 1998). The *ein3* loss-of-function mutants are recessive and insensitive to ethylene, but this insensitivity is not as severe as in the *ein2* mutants, probably due to functional redundancy between EIN3 and the EILs. It has also been

shown that overexpression of *EIN3* leads to a constitutive ethylene response, even in an *ein2* background (Chao et al., 1997). These data, along with the observation that the *ein2*;*ein3* double mutant does not show an additive phenotype (Roman et al., 1995), suggest that EIN3 acts downstream of EIN2 in the ethylene signal transduction pathway. *EIN3* gene expression is not induced by ethylene (Chao et al., 1997), but the EIN3 protein only accumulates in the presence of the hormone (Yanagisawa et al., 2003). EIN3 is necessary and sufficient for the expression of the transcription factor ETHYLENE RESPONSE FACTOR1 (ERF1) (Solano et al., 1998). *ERF1* is a member of a large family of plant-specific transcription factors with more than a hundred members in Arabidopsis. ERF1 is one of the transcription factors responsible for the induction of ethylene response genes but is not accountable for the entire ethylene response (Riechmann and Meyerowitz, 1998; Wang et al., 2002).

The Ethylene Receptor Multigene Family

The focus of this work is to characterize the ethylene receptors and their role in ethylene signal transduction. As shown in Figure 1-3, the ethylene receptors show four distinct domains: a membrane spanning domain, which is the ethylene binding site (Schaller and Bleecker, 1995); a GAF domain (Aravind and Ponting, 1997); a kinase domain with sequence similarity to histidine kinases (Parkinson and Kofoid, 1992); and a receiver domain as found in response regulator proteins (Stock et al., 2000). The receiver domain, however, is absent from two of the ethylene receptors, ERS1 and ERS2 (Hua et al., 1995; Hua et al., 1998).

It has been shown that ETR1 is present as a dimer, held together by a disulfide linkage that is predicted to be extra-cytoplasmic (Schaller et al., 1995). The ethylene binding site is located in the membrane-spanning region of the amino-terminal domain

and competition assays with ^{14}C-labeled ethylene show that ethylene binding in yeast expressing ETR1 is saturable and reversible. Measurements of ^{14}C released from the receptor suggest that the half-life of ethylene binding is 12.5 hours (Schaller and Bleecker, 1995). However, this might be an underestimate as protein turnover was not taken into account. Ethylene seems to interact with the copper atom in the electron rich hydrophobic region within the membrane-spanning domains of the dimer (Rodriguez et al., 1999). The coordination of a copper atom explains the observed reversible ethylene binding to the receptor. Cys65 and His69 are necessary for binding activity, as these residues coordinate the metal. In the *etr1-1* mutant, the C65Y mutation prevents the receptor from coordinating the copper atom, averting ethylene binding (Hall et al., 1999; Rodriguez et al., 1999). The most conserved domain in all ethylene receptors is the trans-membrane (sensor) domain, including the amino acids that are required for dimerization and ethylene binding.

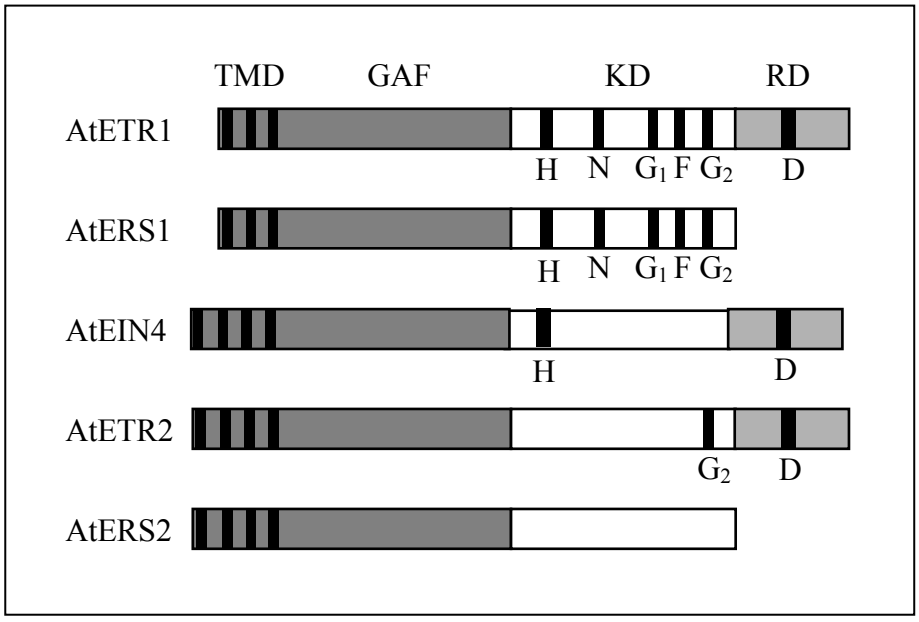

Figure 1-3. Schematic of the ethylene receptor family in Arabidopsis. The five conserved motifs necessary for histidine kinase activity (H-, N-, G1-, F-, and G2-boxes) are noted.

The GAF domain has been identified by sequence comparison, and it is defined by a predicted secondary conformation. This domain was first identified in cGMP-specific phosphodiesterases, adenylate cyclases from *Anabaena*, and FhlA from *E. coli*, and is present in eubacterial and eukaryotic signaling molecules. The function of the GAF domain is not clear, but it seems to be a binding site for ligands, such as nucleotides and small molecules (Aravind and Ponting, 1997). The GAF domain has also been identified as the chromophore-binding domain of plant phytochromes, which also show sequence similarity to bacterial two-component systems (Sharrock and Quail, 1989).

The carboxyl-terminal region of ETR1 shows the main features of two-component regulators. ETR1 contains the five conserved motifs necessary for histidine kinase activity (H-, N-, G1-, F-, and G2-boxes), in the conserved order and with loosely conserved spacing (Figure 1-3) (Parkinson and Kofoid, 1992; Chang et al., 1993). Histidine autophosphorylation activity of ETR1 has been shown at the conserved histidine in the H-box (Gamble et al., 1998). The receiver domain of ETR1 has two of the three conserved aspartate residues, including the one that is phosphorylated, and the conserved lysine. However, no phosphorelay has been observed. It has also been reported that loss of histidine autophosphorylation or removal of the kinase domain of ETR1 does not impair ethylene insensitivity conferred by the dominant *etr1* mutant (Gamble et al., 2002). Neither do mutations that disrupt histidine kinase activity of ETR1 prevent its complementation of *etr1;ers1* ethylene hypersensitive double loss-of-function mutant (Wang et al., 2003). Hence, it has been suggested that receptor kinase activity is not part of the mechanisms of ethylene signal transduction.

The Arabidopsis genes fall into two subfamilies, with respect to their sequence similarity (Figure 1-4). These two groups do not correspond to the presence or absence of the response regulator but they do correlate with intron distribution within the genes. ETR1 and ERS1 are subfamily 1 receptors and have all the conserved motifs necessary for histidine kinase activity (Figure 1-3) (Parkinson and Kofoid, 1992; Chang et al., 1993; Hua et al., 1995). The subfamily 2 class includes ETR2, ERS2 and EIN4, which do not contain most of the motifs characteristic of histidine kinases (Hua et al., 1998; Sakai et al., 1998). EIN4 is the only one in this group containing a histidine in the same position as the one that is phosphorylated in two-component and phosphorelay systems (Hua et al., 1998).

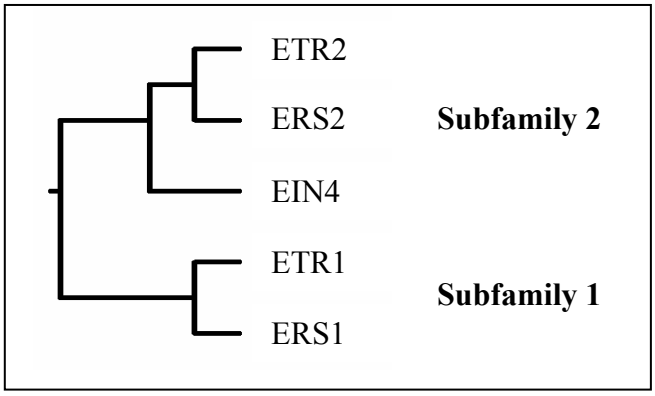

Figure 1-4. Phylogenetic relationship between the Arabidopsis ethylene receptors. Neighbor-joining tree was generated from full-length protein sequences using Clustal W (http://clustalw.genome.ad.jp/).

The subfamily 2 members feature a putative fourth trans-membrane region at the amino-terminus of 20-30 hydrophobic amino acids, which could be a targeting sequence (Hua et al., 1998; Sakai et al., 1998). The ethylene receptors also show differential gene regulation: *ETR2*, *ERS1*, and *ERS2* are induced by ethylene while the others are not. *In situ* hybridization data suggest that these genes are expressed in all organs, in

overlapping, but not identical, tissues (Hua et al., 1998). Despite the divergence in sequence, genetic data suggest that all family members are active in ethylene signal transduction. Dominant ethylene insensitive mutations have been recovered for all ethylene receptors (Bleecker et al., 1988; Hua et al., 1995; Hua et al., 1998; Sakai et al., 1998) and single loss of function mutants do not lead to a constitutive ethylene response phenotype (Hua and Meyerowitz, 1998; Hall et al., 1999).

In tomato, six ethylene receptor family members have also been identified: LeETR1, LeETR2 (Lashbrook et al., 1998), NR (Wilkinson et al., 1995), LeETR4, LeETR5 (Tieman and Klee, 1999), and LeETR6 (Tieman and Klee, unpublished). *Nr* mutants show several ethylene insensitive phenotypes, such as absence of seedling triple response in the presence of ethylene and delayed tomato fruit ripening, abscission and senescence (Yen et al., 1995). The negative regulation model is supported by receptor studies in tomato, where antisense lines for *LeETR4* show a severe ethylene response phenotype. Interestingly, antisense lines for other ethylene receptors, such as *NR*, do not seem to show ethylene response phenotypes, as *LeETR4* seems to be upregulated in these lines, which compensates for the reduction in *NR* receptor levels (Tieman et al., 2000). Compensation for lack of *LeETR4* does not occur, as a result of differential regulation of gene expression. However, the ethylene hypersensitive response phenotype can be eliminated in these *LeETR4* antisense lines by the overexpression of *NR*. These data support the hypothesis that the receptors perform redundant functions and also suggest that the response regulator is not necessary for the ethylene response signal transduction pathway, as it is absent from the *NR* protein.

CHAPTER 2
EXPERIMENTAL PROCEDURES

This chapter outlines the experimental procedures used to obtain the results presented in the next few chapters. The descriptions of the methods used in this work are moderately detailed and they assume the reader has at least a basic knowledge of biochemistry and molecular biology. Previously published techniques that were followed as described in the original work (or kit manual) are only referenced, while all modifications and optimizations made to those are described in detail.

Experimental Procedures for Tomato Receptor Studies (Chapter 3)

The genomic sequence between the translational start and stop sites of the tomato ethylene receptors *LeETR2*, *NEVER RIPE* (*NR*), *LeETR4*, *LeETR5*, and *LeETR6* was isolated from *Lycopersicon esculentum* and cloned into an expression vector for localization studies. These constructs permitted the expression of the tomato receptors *in vivo* with the enhanced green fluorescent protein (EGFP) attached to their carboxyl-termini. Two kilobases of the promoter region of *NR* was also isolated from *L. esculentum* and cloned into an expression vector where it controlled the expression of β-glucuronidase (GUS). This construct was used to produce transgenic tomato plants for expression studies.

Isolation of Genomic Sequences for the Tomato Ethylene Receptors

Genomic DNA was isolated from tomato leaves (*Lycopersicon esculentum cv. M82*) using the DNeasy Plant Mini Kit (Qiagen), according to manufacture's guidelines.

Genomic DNA was used to amplify the genomic loci of the tomato ethylene receptors, as described below. Primers used for cloning are described in Table 2-1 and sequencing primers are described in Table 2-2. The PCR products were then cloned into the pENTR/D-TOPO vector (Invitrogen), which contains the site-specific recombination sites *att*L1 and *att*L2. Forward primers were designed incorporating the CACC nucleotide sequence (underlined) required for directional cloning into pENTR/D-TOPO.

Table 2-1. Cloning primers for the tomato genomic sequences*

Primer Name	**Primer Sequence**
LeETR2-fwd3	GTGATTCATTAAGGATTTGTTCATCATGGATTGTA
LeETR2-rev3	TTGAGACAATTTTGGTTTACTGGGATTAAAGAACAGT
LeETR2-entry5'	CACCATGGATTGTAACTGCTTCGATCCACTGTTG
LeETR2-rev2	AAGAACAGTTCCGTGCTCTAAAAGCCCGGATA
NR-entry5'	CACCATGGAATCCTGTGATTGCATTGAGGCTTTAC
NR-rev2	CAGACTTCTTTGATAGCGTTGAGCATTCACAGAC
LeETR4-entry5'	CACCATGTTGAGGACGTTAGCATCAGCTTTGTTG
LeETR4-rev2	CATCATTCTACTTCCCCGTAGCAGAACCCTTT
LeETR5-fwd3	GGATTGAGATGTTGGCAATGTTAAGGTTGTTG
LeETR5-rev3	CATTAGTACTAACATCTCACAAGCCATCACCACC
LeETR5-entry5'	CACCCACCATGTTGGCAATGTTAAGGTTGTTGTTTCT
LeETR5-rev2	CAAGCCATCACCACCGCCGC
LeETR6-fwd3	GGTGTAAACAAGAGTAGTTCTATTGGATGCAATGATGAAG
LeETR6-rev3	TATAGTCTATTGTAAACGTTACCGTCATGGCATTCCT
LeETR6-entry5'	CACCATGATGAAGAAAGTAGTATCATGGTTGTTGTT
LeETR6-rev2	TGGCATTCCTCTGTTTGCATG
EGFP-*Spe* I-fwd	ACTAGTATGGTGAGCAAGGGCGAGGAG
EGFP-*Kpn* I-rev	CCATGGAATGAACATGTCGAGCAGGTACGGCTCT

*Underlined nucleotides are explained in the text.

LeETR2. *LeETR2* was amplified by nested PCR with the following conditions: primers LeETR2-fwd3 and LeETR2-rev3 were used to amplify genomic DNA using the following cycle conditions: 5 min at 95°C, 30 cycles of 95°C for 1 min, 61°C for 1 min, and 72°C for 8 min, followed by 10 min at 72°C. Genomic PCR products were used in a nested PCR reaction with primers LeETR2-entry5' and LeETR2-rev2 using the following cycle conditions: 5 min at 95°C, 30 cycles of 95°C for 1 min, 61°C for 1 min, and 72°C for 8 min, followed by 10 min at 72°C. *LeETR2* sequence was obtained by automated sequencing with the following primers: M13F (Invitrogen), M13R (Invitrogen), LeETR2-

seqfwd-1, LeETR2-seqfwd-2, LeETR2-seqfwd-3, LeETR2-seqrev-1, LeETR2-seqrev-2, LeETR2-seqrev-3, LeETR2-seqrev-4, LeETR2-seqrev-5, LeETR2-seqrev-6, 7-3-2 (rev), 7-3-4 (rev), 7-3-5 (fwd), 7-3-6 (fwd), and 7-3-8 (rev).

NR. *NR* was amplified by genomic PCR with primers NR-entry5' and NR-rev2 using the following cycle conditions: 5 min at 95°C, 30 cycles of 95°C for 1 min, 63°C for 1 min, and 72°C for 7 min, followed by 10 min at 72°C. *NR* sequence was obtained by automated sequencing with the following primers: M13F, M13R, NR-seqfwd-1, NR-seqfwd-2, NR-seqfwd-3, NR-seqfwd-4, NR-seqfwd-5, NR-seqrev-1, NR-seqrev-2, NR-seqrev-3, and NR-seqrev-4.

LeETR4. *LeETR4* was amplified by genomic PCR with primers LeETR4-entry5' and LeETR4-rev2 using the following cycle conditions: 4 min at 95°C, 30 cycles of 95°C for 1 min, 63.4°C for 1 min, and 72°C for 7 min, followed by 15 min at 72°C. *LeETR4* sequence was obtained by automated sequencing with the following primers: M13F, M13R, LeETR4-seqfwd-1, LeETR4-seqfwd-2, LeETR4-seqrev-1, T12-1-3 (fwd), T12-1-4 (fwd), T12-1-5 (rev), and T12-1-8 (rev).

LeETR5. *LeETR5* was amplified by nested PCR with the following conditions: primers LeETR5-fwd3 and LeETR5-rev3 were used to amplify genomic DNA using the following cycle conditions: 4 min at 95°C, 30 cycles of 95°C for 1 min, 62°C for 1 min, and 72°C for 7 min, followed by 15 min at 72°C. Diluted genomic PCR products were used in a nested PCR reaction with primers LeETR5-entry5' and LeETR5-rev2 using the following cycle conditions: 2 min at 95°C, 30 cycles of 95°C for 30s, 60.3°C for 30s, and 72°C for 10 min, followed by 15 min at 72°C. *LeETR5* sequence was obtained by automated sequencing with primers M13F, M13R, LeETR5-seqfwd-1, LeETR5-seqfwd-

2, LeETR5-seqfwd-3, LeETR5-seqfwd-4, LeETR5-seqfwd-5, LeETR5-seqrev-1, LeETR5-seqrev-2, LeETR5-seqrev-3, T9-2-2 (fwd), T9-2-6 (rev), and T9-2-9 (fwd).

Table 2-2. Sequencing primers for tomato genomic clones.

Primer Name	**Primer Sequence**
LeETR2-seqfwd-1	CTCCCTGTAATTAATCAAGT
LeETR2-seqfwd-2	CTGGTCGCTTAAATGAGTCA
LeETR2-seqfwd-3	TCATCTGCACCACGCTGA
LeETR2-seqrev-1	CGTTACCATCCTGCTAAACC
LeETR2-seqrev-2	GCTTCCATCCTCTAGCCTTG
LeETR2-seqrev-3	GCCAAGTCCAGTGCCAGCAG
LeETR2-seqrev-4	TGACGGCAACATCATAACAG
LeETR2-seqrev-5	GTTAAGCTCCTTCAACTTTC
LeETR2-seqrev-6	TAGATCCTACATCCAACGTC
7-3-2 (rev)	CAAAGCATAGCTCTTCGG
7-3-4 (rev)	CCCTAACAGCAACCACCTC
7-3-5 (fwd)	ACTTCTATTTGCGTGTACAGG
7-3-6 (fwd)	GAGCAGAATGTGGCTCTTGATC
7-3-8 (rev)	CCGAAGACAAACTAAGCGTGAC
NR-seqfwd-1	TCCTTTGGCAAGGATGAGGA
NR-seqfwd-2	GCAGTTATTGCTCTGTGCTC
NR-seqfwd-3	CCAAACTCTCTTAAACGTGGC
NR-seqfwd-4	GAGATAAGAAGCACACTCGA
NR-seqfwd-5	GCTCAGACTCTGGTGTCG
NR-seqrev-1	AATTTGGTGAATACTAGTGG
NR-seqrev-2	GAGCACAGAGCAATAACTGC
NR-seqrev-3	GCCACGTTTAAGAGAGTTTGG
NR-seqrev-4	CGCACCTAAACATCCTTACC
LeETR4-seqrev-1	CGAAGCCATCCAAATCAGGC
T12-1-3 (fwd)	CAGGTTAAGGAGAGTGACGGAG
T12-1-4 (fwd)	GGCATTGGTGGAGCATGGAG
T12-1-5 (rev)	GCAACAAGGAGAGCAGACCC
T12-1-8 (rev)	CCCAGATGTCTCCTTGCATCAAC
LeETR5-seqfwd-1	CTTTCCAATTGATATTGTCC
LeETR5-seqfwd-2	CTTGAGTTTCAACATGTGCA
LeETR5-seqfwd-3	GAGAAAGAAGGGAGGAAC
LeETR5-seqfwd-4	CCATGAAGAGACTGACTACC
LeETR5-seqfwd-5	TTGAGCTGTAGGAACCAGTC
LeETR5-seqrev-1	TCTGAGAATGAGTGTCATCC
LeETR5-seqrev-2	TGCACATGTTGAAACTCAAG
LeETR5-seqrev-3	CTGTCCGCAGAAGCAGTA
T9-2-2 (fwd)	CACTTATTAAATGTCAGCATTGG
T9-2-6 (rev)	CTAACCTCAATTTCAAATTTTATGG
T9-2-9 (fwd)	GAGCTAGATCAAGAGGTTGGG
LeETR6-seqfwd-1	TTAGGATGCTTACCAATGAG
LeETR6-seqfwd-2	TTAGAGCAAGTCAAGCTAGGA
LeETR6-5 (fwd)	CCGAGATCGAACTCATCCAATG

LeETR6. *LeETR6* was amplified by nested PCR with the following conditions: primers LeETR6-fwd3 and LeETR6-rev3 were used to amplify genomic DNA using the following cycle conditions: 5 min at 95°C, 30 cycles of 95°C for 1 min, 63°C for 1 min, and 72°C for 3 min, followed by 10 min at 72°C. Diluted genomic PCR products were used in a nested PCR reaction with primers LeETR6-entry5' and LeETR6-rev2 using the following cycle conditions: 1 min at 95°C, 30 cycles of 95°C for 30s, 57°C for 30s, and 72°C for 3 min, followed by 5 min at 72°C. *LeETR2* sequence was obtained by automated sequencing with the following primers: M13F, M13R, LeETR6-seqfwd-1, LeETR6-seqfwd-2, and LeETR6-5 (fwd).

Cloning of the Tomato Ethylene Receptors for Localization Studies

The genomic clones of the tomato ethylene receptors cloned into pENTR/D-TOPO were recombined into a destination vector (pDESTOE-GFP; Figure 2-1).

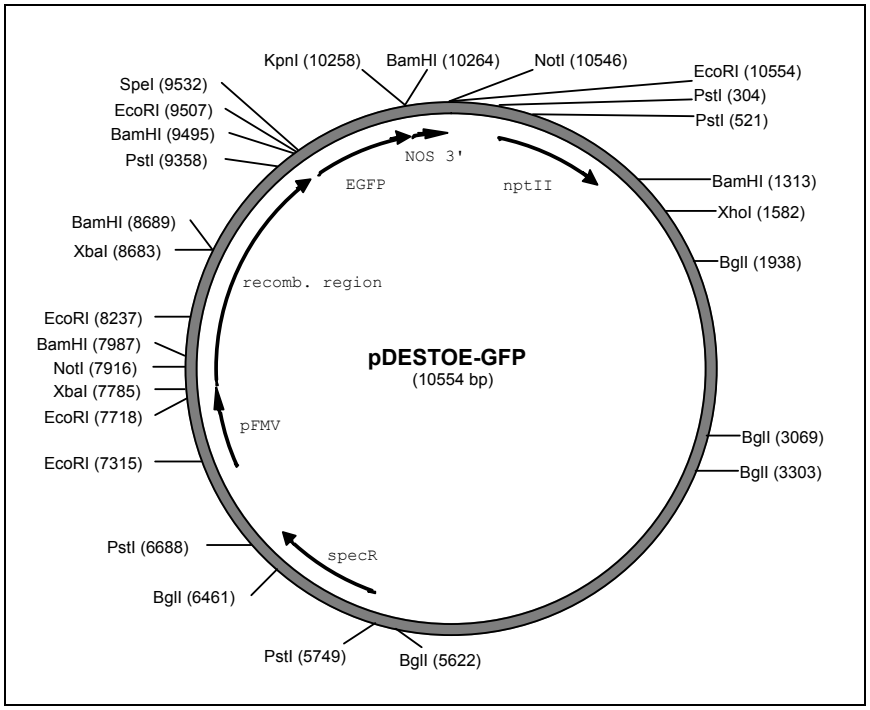

Figure 2-1. The pDESTOE-GFP vector is a plant transformation vector containing the EGFP sequence downstream of an engineered recombination region.

The pDESTOE-GFP vector contains the gene for enhanced green fluorescent protein (EGFP) and expresses a fusion protein with the EGFP at the carboxyl-termini of the receptors. This vector was created as follows: the EGFP gene was amplified from pGREEN0029 (John Innes Centre, United Kingdom) with primers EGFP-*Spe* I-fwd and EGFP-*Kpn* I-rev (Table 2-1), with engineered restriction sites to facilitate subcloning (underlined). The PCR conditions used were: 30 cycles of 95°C for 30s, 60°C for 30s, and 72°C for 1 min. The EGFP PCR product was cloned into pCR4-Blunt-TOPO (Invitrogen), and sequenced with M13F and M13R primers. The EGFP fragment was then cut with *Spe* I and *Kpn* I and ligated into the destination vector pDESTOE (Figure 2-2) cut with the same endonucleases.

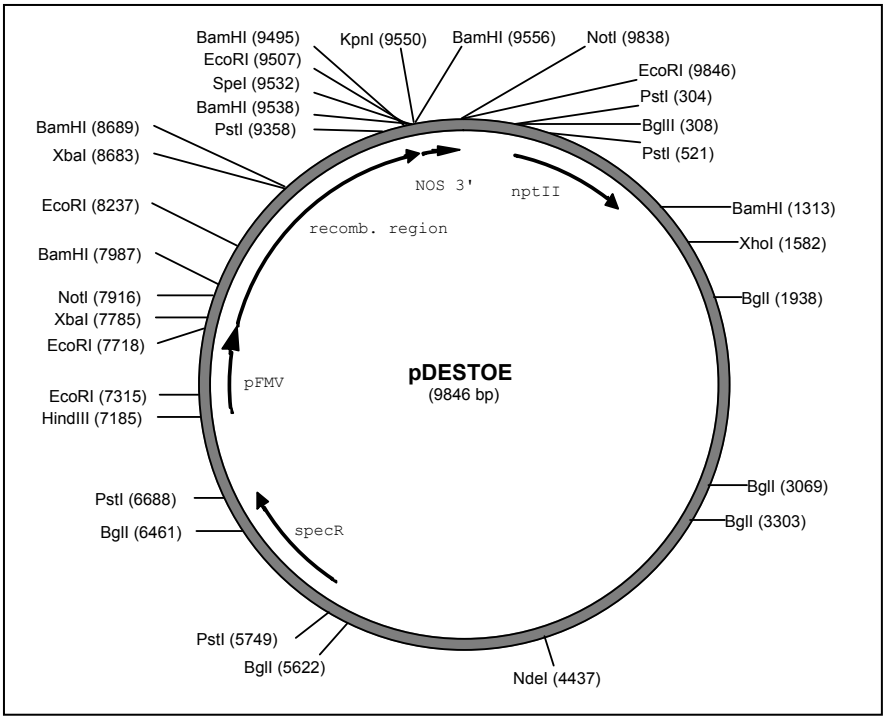

Figure 2-2. The pDESTOE vector is a plant transformation vector with an engineered recombination region.

The pDESTOE vector was made by ligating the Gateway Cassette (Invitrogen) into pFMV-nos (Figure 2-3) between the FMV-35S promoter and the nos terminator. The *Not*

I-*Not* I fragment was then cloned into a standard vector for *Agrobacterium*-mediated transformation (pHK1001; Figure 2-4).

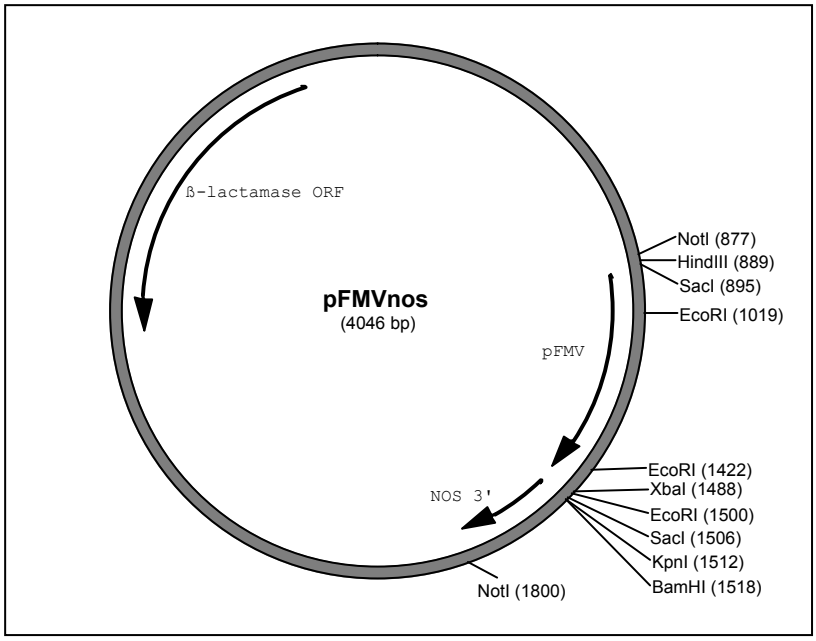

Figure 2-3. The pFMVnos vector contains the FMV-35S promoter and the nos terminator.

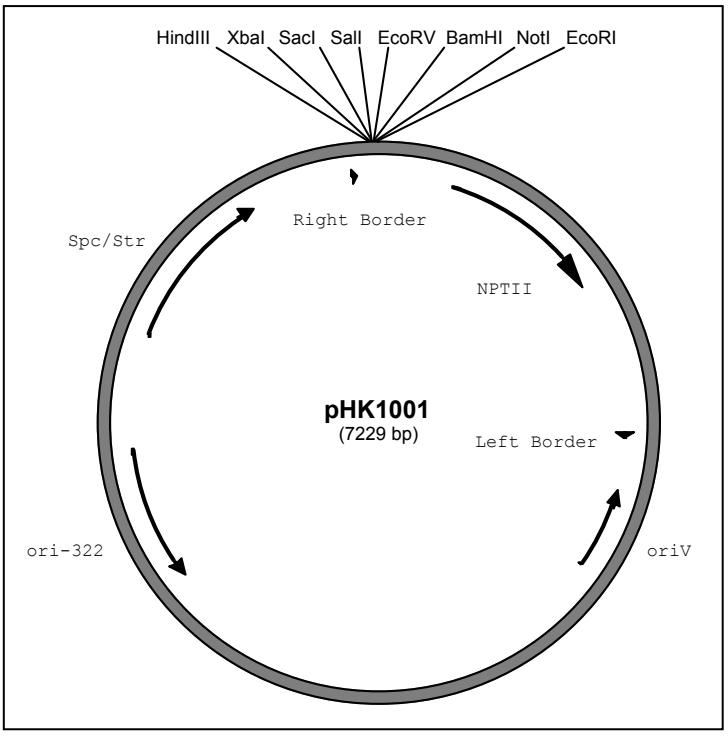

Figure 2-4. The pHK1001 vector is a standard plant transformation vector.

Protoplast Isolation and Transient Expression of EGFP Fusion Proteins

This procedure was performed by Isabelle Mila in Dr. Mondher Bouzayen's laboratory at UMR 990, INRA-ENSAT- Genomique et biotechnologie des fruits, France. Protoplasts used for transfection were obtained from 6- to 8-day-old suspension–cultured tobacco BY-2 cells. For the cell wall digestion, ~2 g of cells were first rinsed two times with a Tris-MES (25 mM) buffer containing 0.6 M mannitol, pH 5.5, and then incubated for 1.5 h at 37°C in the same solution containing 1% Caylase, 0.2% pectolyase Y-23 and 1% BSA. Protoplasts were then filtered through 30 μm nylon cloth and washed three times with W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose and 0.1% MES, pH 5.6). Protoplasts were transfected with the receptor:EGFP constructs by a modified polyethylene glycol method (Abel and Theologis, 1994). Typically, 0.2 ml of protoplast suspension (0.5 x 10^6/ml) was transfected with 50 μg of sheared salmon sperm carrier DNA (Clontech) and 20 μg of the appropriated plasmid DNA. Transfected protoplasts were incubated at 25°C for 16 hours. Confocal fluorescent images were obtained on a confocal laser scanning microscope (Leica TCS SP2, Leica DM IRBE ; Leica Microsystems, Wetzlar, Germany). The samples were illuminated with an argon ion laser (488 nm wavelength) for GFP. The emitted light was collected at 500-525 nm.

Isolation of the *NR* Promoter Sequence

Genomic DNA was isolated from tomato leaves (*L. esculentum cv. Floridade* and *cv. Pearson*) using the DNeasy Plant Mini Kit (Qiagen), according to manufature's guidelines. Genomic DNA from Floridade and Pearson cultivars were used to make GenomeWalker libraries according to the Universal GenomeWalker kit user manual (Clonetech). In brief, genomic DNA was digested overnight with five blunt-cutting restriction endonucleases (*Dra* I, *EcoR* V, *Pvu* II, *Sca* I and *Stu* I), purified by

phenol/chloroform extraction and ethanol precipitation, and ligated to GenomeWalker adaptors.

GenomeWalker libraries were screened by PCR, according to manufacturer's guidelines, using *NR* sequence-specific primers (Table 2-3) and adapter primers provided with the kit. The sequence-specific primers used for isolation of the *NR* promoter were: NR-1, NR-2 (nested), NR-3, NR-4 (nested), NR-5, and NR-6 (nested). The PCR conditions were as follows: 7 cycles of 94°C for 20s and 70°C for 3 min, 37 cycles of 94°C for 20s and 65°C for 3 min, followed by 7 min at 65°C. Diluted PCR products were subjected to a nested PCR with *NR* sequence-specific primers and nested adapter primers provided with the kit. The nested PCR conditions were as follows: 5 cycles of 94°C for 20s and 70°C for 3 min, 20 cycles of 94°C for 20s and 70°C for 3 min, followed by 7 min at 67°C. PCR products from nested PCR were cloned into the *Srf* I site of pPCR-Script Amp SK(+) plasmid (Stratagene) and sent for automated sequencing. Obtained sequence was used to design new sequence-specific primers, which were then used to screen the existing GenomeWalker libraries. This procedure was repeated three times in order to isolate 2.1 kb of promoter sequence for *NR*.

Table 2-3. Primers used to isolate and clone the *NR* promoter*

Primer Name	**Primer Sequence**
NR-1	TGCTATTTCCTGCTGCGACACATACCTGTC
NR-2 (nested)	GACGACGGAGAATGCGATCTCAGTATCTAC
NR-3	CATGATCACCGAGAATATTAGTAGCTCAG
NR-4 (nested)	AATTCCGAACATGTAGCGTTTTCATCC
NR-5	CGGAATTTTAGTTGAAACTTACAGGGTTACC
NR-6 (nested)	GAACACAAACCTATGGACTCAGCAAAAGC
NR-promt-5'-2	CTAAAAGGGGGATTAGTTCTTATTTTTAAT
NR-promt-3'short	CCATGGGATTTTCGTCGTGTTCTTCG

*Underlined sequence is the engineered *Nco* I site.

Cloning of the *NR* Promoter Sequence for Expression Studies

The *NR* promoter sequence was amplified from Pearson genomic DNA with the primers NR-promt-5'-2 and NR-promt-3'short (Table 2-3), using the following cycle conditions: 35 cycles of 95°C for 45s, 60°C for 45s, and 72°C for 4 min. *NR* promoter PCR product was cloned into the *Srf* I site of pPCR-Script Amp SK(+) plasmid (Stratagene) to produce plasmid pPM7. The *NR* promoter was cut from pPM7 with *Not* I and *Nco* I, and subcloned into pMON637 (Monsanto Co.), which contained the coding sequence for GUS, to produce plasmid pPM8. The *NR* promoter-GUS fusion segment was cut from pPM8 with *Not* I and cloned into a *Not* I site in the pHK1001 expression vector, which contained the NPTII gene for spectinomycin resistance, to produce plasmid pPM9. Plasmids were purified using either Plasmid Midi Prep kit (Stratagene) or Plasmid Maxi Prep kit (Qiagen).

Tomato Transformation

This procedure was performed by Dr. Mark G. Taylor in Dr. Harry J. Klee's laboratory at the University of Florida. Transgenic NR-GUS tomato plants (*Lycopersicon esculentum* cv. *Micro-Tom*) were produced by *Agrobacterium*-mediated transformation (Mccormick et al., 1986) of pPM9, using spectinomycin resistance as a selectable marker. Introduction and inheritance of the transgene were confirmed by PCR using primers specific for the selectable marker.

GUS Activity Assay

Histochemical staining for GUS activity was performed by treating dissected tissue in assay solution (0.1 M $NaPO_4$ buffer (pH 7.0), 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM $K_4[Fe(CN)_6]$, 10 mM Na_2EDTA, 0.1% (w/v) 5-bomo-4-chloro-3-indolyl-β-D-gluguronic acid (X-GLUC)(BioVectra), 0.1% (v/v) TritonX-100) for 24h at 37°C. Tissue was rinsed

twice in 80% (v/v) ethanol and incubated at 4°C in ethanol to remove chlorophyll pigment. Stained tissue was visualized through a Wild dissecting stereomicroscope. Images were captured with a Leica DC300 color CCD camera and imported into Adobe Photoshop using a Leica twain driver.

Experimental Procedures for Arabidopsis Expression Studies (Chapter 4)

Ethylene receptor messenger RNA (mRNA) levels were quantified by real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR), using total RNA isolated from different wild-type and mutant lines of *Arabidopsis thaliana*. Exogenous ethylene treatments were performed for 1h in sealed glass chambers containing 10 ppm C_2H_4.

RNA Isolation

Total RNA was extracted from 1 g of rosette tissue in extraction buffer (1% (w/v) triisopropylnaphthalene-sulfonic acid (TIPS), 6% (w/v) ρ-amino salicylic acid, 100 mM Tris (pH 8.0), 50 mM EGTA, 0.1 M NaCl, 1% (w/v) SDS, 0.039% (v/v) β-mercaptoethanol) containing 50% (v/v) phenol:chloroform:isoamyl alcohol (PCI) solution (25:24:1 (v/v/v)). The extraction mixture was homogenized with a polytron and incubated 20 min at 50°C. The phases were separated by centrifugation and an equal volume of PCI was added to the aqueous phase. The RNA was ethanol precipitated overnight and purified by two consecutive LiCl (2 M) precipitations. Purified RNA was ethanol precipitated and pellets were resuspended in water treated with diethyl pyrocarbonate (DEPC). RNAs were then treated with DNaseI (Ambion), followed by removal of impurities with RNeasy RNA extraction kit (Qiagen) according to the manufacturer's instructions.

Real Time RT-PCR (TaqMan)

Ethylene receptor mRNA levels were quantified by Real-time quantitative RT-PCR using Taqman one-step RT-PCR reagents (Applied Biosystems) and an Applied Biosystems GeneAmp 5700 sequence-detection system. Each determination was performed using 250 ng of *DNase* I-treated total RNA, in a 25-µl reaction volume. RT-PCR conditions were: 48°C for 30 min, 95°C for 10 min followed by 40 cycles of 95°C for 15s and 60°C for 1 min. Absolute mRNA levels were quantified using custom-made standard curves. The sense strand transcripts used to generate the standard curves were synthesized using an *in vitro* transcription kit (Ambion), via the incorporation of [^{3}H] UTP using T7 or T3 RNA polymerase. Lengths of transcribed sense probes were 1267 nt (ETR1), 1887 nt (ERS1), 1908 nt (ETR2), 1460 nt (ERS2) and 2416 nt (EIN4).

Primers and probes were designed using Primer Express software (Applied Biosystems) and were as follows: ETR1 fwd primer, ETR1 rev primer, ETR1 Taqman probe, ETR2 fwd primer, ETR2 rev primer, ETR2 Taqman probe, EIN4 fwd primer, EIN4 rev primer, EIN4 Taqman probe, ERS1 fwd primer, ERS1 rev primer, ERS1 Taqman probe, ERS2 fwd primer, ERS2 rev primer, and ERS2 Taqman probe. Primers and probes are described in Table 2-4.

Arabidopsis Infections with *Xanthomonas campestris*

This procedure was performed by Dr. Phillip J. O'Donnell in Dr. Harry J. Klee's laboratory at the University of Florida. As described previously (O'Donnell et al., 2003), *Arabidopsis thaliana* Columbia (Col-0), *etr1-1* (Bleecker et al., 1988) and *etr2-1* (Sakai et al., 1998) and the NahG line (Novartis) were grown in soil under long night conditions (8h day; 16h night) for 6 weeks to encourage vegetative growth. Forty-eight hours prior to treatment, plants were transferred to a 16h day, 8h night regime, and 12h before

infection were enclosed in a humidity dome to aid bacterial entry. Plants were inoculated with *Xanthomonas campestris* pv. *campestris* (*Xcc*) strain 33913 by first submerging the whole plant in a suspension of 5 x 10^6 CFU bacteria, containing 10 mM $MgCl_2$ and 0.02% (v/v) Silwet L-77, for 30s. A vacuum was then applied to the soaked plants for 1 min to aid bacterial entry and the plants were returned to the humidity chamber overnight.

Table 2-4. Primers and probes used for real-time RT-PCR assays*

Primer Name	**Primer Sequence**
ETR1 fwd primer	TGAGTTGATTTACTTTGTGAAGAAATCA
ETR1 rev primer	GTTGCTCCACAAAGAACGATAAAA
ETR1 Taqman probe	6FAM-CTGAACAAGTACCCATCTATACGGAAACACGG-TAMRA
ETR2 fwd primer	TTAGCTATAACGGCGGTGGTT
ETR2 rev primer	GAATGTTCTCTGTACTCCAGAAACTGTT
ETR2 Taqman probe	6FAM-CCTTCGTCTTCGCAGTTACATCGTGGA-TAMRA
EIN4 fwd primer	CTTTAGGTCTTGGATTGCTTCTGTT
EIN4 rev primer	GAAACCTTCGTCGTCACAATTACA
EIN4 Taqman probe	6FAM- TCACGTAATCGTTATCACCAGAAACCAGAGCA-TAMRA
ERS1 fwd primer	CAACCTTTATGGATGTTCTTCATGCA
ERS1 rev primer	CACAACCGCGCAAGAGACT
ERS1 Taqman probe	6FAM-CCAAAGCCGTTGCCATTGTCATGA-TAMRA
ERS2 fwd primer	GCAGAAGACGACGGTAGCTTGT
ERS2 rev primer	CGATAAGAAAGTCGCCGACTTT
ERS2 Taqman probe	6FAM-TCTTTAGCTACGAGACAATCCTCAACTCGCA-TAMRA

*6FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

Experimental Procedures for Enzyme Assays (Chapter 5)

In order to produce recombinant proteins suitable for *in vitro* enzyme assays, only the soluble domains of the Arabidopsis ethylene receptors were used. The constructs included the GAF domain, the kinase domain and the receiver domain, when the latter was present in the native protein, but lacked the amino-terminal membrane-spanning domain. The soluble domains of all five Arabidopsis ethylene receptors were expressed in the yeast *S. pombe*, each with a GST tag attached to its amino-terminus. The purified recombinant proteins were then assayed for autophosphorylation activity *in vitro*.

Construction of Yeast Expression Plasmids

The soluble domains of the Arabidopsis ethylene receptors were amplified from cDNA clones with the following primers (engineered restriction sites are underlined): ETR1-ESP-fwd and ETR1-ESP-rev; ETR2-ESP-fwd and ETR2-ESP-rev; ETR2-ΔGAF-fwd and ETR2-ΔGAF-rev; ESR1-ESP-fwd and ESR1-ESP-rev; ERS2-ESP-fwd and ERS2-ESP-rev; and EIN4-ESP-fwd and EIN4-ESP-rev. Primers are described in Table 2-5. ETR1, ERS1 and EIN4 PCR products were cut with *BamH* I and cloned into pESP-1 (Stratagene), ETR2 and ETR2-GAF were cut with *Xma* I and cloned into pESP-1, and ERS2 was cut with *Bgl* II and cloned into the *BamH* I site of pESP-1.

Table 2-5. Primers used for kinase assay constructs*

Primer Name	Primer Sequence
ETR1-ESP-fwd	AGCTCGGATCCGAAATGGGATTGATTCGAACTCA
ETR1-ESP-rev	ATCCAGGATCCTTACATGCCCTCGTACAGTACC
ETR2-ESP-fwd	GAGCTTCCCGGGGAAGTTGGTTTGATTTTGATTAA
ETR2-ESP-rev	AGCCATCCCGGGTTAGAGAAGTTGGTCAGCTTGCAAC
ETR2-ΔGAF-fwd	ATGGCGCCCGGGGACGCGTTGAGAGCGAGCCAAGC
ETR2-ΔGAF-rev	AGCCATCCCGGGTTAGAGAAGTTGGTCAGCTTGCAAC
ESR1-ESP-fwd	AGTTAGGATCCGAAATGGGTCTTATTTTAACACA
ESR1-ESP-rev	TCCATGGATCCTCACCAGTTCCACGGTCTGGTTTGT
ERS2-ESP-fwd	AGAGCTTAGATCTGAGGTTGGGATCATTATGAAGCA
ERS2-ESP-rev	CATGGATAGATCTTCAGTGGCTAGTAGACGGAGGAGTT
EIN4-ESP-fwd	AGCTTGGATCCGAGGTTGGATTGATGAAGAGGCA
EIN4-ESP-rev	AGGATGGATCCTCACTCGCTCGCGGTCTGCAAAGC
ETR1-H-fwd	GAACACCGATGGCTGCGATTATTGCACT
ETR1-H-rev	GCATTTCAGCGTTCATAACCGCTAG
ETR2-H-fwd	CCTATGGCTTCGATACTCGGTCTTT
ETR2-H-rev	ACGCCTCATCCCTTCGCTCATCGTT
ERS1-H-fwd	GGACACCGATGGCTGCCATCATCTCTCT
ERS1-H-rev	TCATCTCGGCGTTCATAACAGCTAG
EIN4-H-fwd	GGAGACCAATGGCCACAATTCTTGGTCT
EIN4-H-rev	TCATTCCAGCACTCATCACTTTCTG
ETR1-G1-fwd	CAGCAGCAATAAATCCTCAAGAC
ETR1-G1-rev	CAGAGTCTTTTACCTTCACTATA
ERS1-G1-fwd	CGTGTGCAATTCACACACAAGAC
ERS1-G1-rev	CTGTGTCCTTCACCTGCACAC

*Underlined nucleotides are explained in the text.

For the H→A and G→A mutations, we used the ExSite Site Directed Mutagenesis kit (Stratagene) on a *BamH* I-*BamH* I fragment containing receptor coding sequence, cut

from the previously described plasmids, cloned into pBSKS(+). The plasmids were methylated prior to mutagenesis. The primers used for the mutagenesis were (nucleotide substitutions are underlined): ETR1-H-fwd and ETR1-H-rev; ETR2-H-fwd and ETR2-H-rev; ERS1-H-fwd and ERS1-H-rev; EIN4-H-fwd and EIN4-H-rev; ETR1-G1-fwd and ETR1-G1-rev; and ERS1-G1-fwd and ERS1-G1-rev. Primers are described in Table 2-5. The reverse primers (rev) were phosphorylated and the cycle parameters for the mutagenesis followed the manufacturer's guidelines. Mutagenesis was confirmed by sequencing and the mutated *BamH* I-*BamH* I fragment was returned to the expression vector.

Recombinant Protein Expression in Yeast

The recombinant constructs were transformed into *S. pombe* SP-Q01 (Stratagene), according to the ESP® Yeast Protein Expression and Purification System protocol (Stratagene). Colonies that grew on agar plates of Edinburgh minimal media (EMM) supplemented with thiamine were selected for screening. Colonies were grown in EMM for 8h and lysed with the Yeast-Buster kit (Novagen), according to manufacturer's protocol. Protein blots of total lysate with a goat anti-GST antibody (Amersham) were used to determine expression levels of positive clones. Expressing clones were grown in 50 ml yeast extract supplemented (YES) media for 18h ($3.0 \leq OD_{600} \leq 4.0$) and used to inoculate 50 ml YES media to an OD_{600} of 0.4. After a 5 hour growth period ($OD600 \approx 1.0$) the cells were washed twice with 50 ml sterile water and resuspended in 500 ml EMM ($OD_{600} \approx 0.1$). The culture was incubated at 30°C for 18-22h ($1.8 \leq OD_{600} \leq 2.2$). Proteins were extracted by vortexing at 4°C with 1X PBST (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 1% (v/v) Triton® X-100) with glass beads (425-600 microns, Sigma) and protease inhibitors: 1 mM PMSF (Sigma), 1 μg/ml aprotinin

(Sigma), 1 μg/ml chymostatin (Sigma), 10 μl/ml protease inhibitor cocktail for fungal and yeast extracts (Sigma). Recombinant proteins were purified from clarified lysate on a Glutathione Sepharose 4B (Amersham) column, which was washed with 1X PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). GST-tagged proteins were eluted with Elution Solution (10 mM reduced glutathione (Sigma), 50 mM Tris-HCl (pH 8.0), 20% (v/v) glycerol). For proteins purified without the GST tag, instead of Elution Solution, columns were eluted with Thrombin (Amersham) in 1X PBS. Eluted proteins were concentrated with Centriplus YM-50 (Amicon) and the buffer exchanged for Storage Solution (50 mM Tris-HCl (pH 8.0), 25% (v/v) glycerol). Purification of recombinant protein was confirmed by protein blot, using goat anti-GST (Amersham) or mouse anti-Flag® (Stratagene) antibodies. Proteins were aliquoted and stored at -80oC.

***In vitro* Autophosphorylation Assays**

Purified recombinant protein (50 pmol) was assayed in 50 mM Tris pH 7.5, 10 mM $MgCl_2$ (or $MnCl_2$), 2 mM DTT, 10% (v/v) glycerol, 0.5 mM [γ-^{32}P] ATP (1 Ci/mmol ≈ 1500 cpm/pmol). The reaction buffer with both Mg^{2+} and Mn^{2+} contained 0.15 mM $MnCl_2$ and 10 mM $MgCl_2$. Mg^{2+} and Mn^{2+} concentrations in solution were calculated by a BASIC version of the COMICS program by (Perrin and Sayce, 1967) using the stability constants described in (O'Sullivan and Smithers, 1979). The reaction buffer for the autophosphorylation of CDPKα contained 0.12 mM $CaCl_2$ and 10 mM $MgCl_2$. Reactions were incubated for 60 min at 25^{o}C and stopped by adding 5X loading dye (250 mM Tris-HCl (pH 6.8), 500 mM DTT, 10% (w/v) SDS, 0.5% (w/v) Bromophenol Blue, 50% (v/v) glycerol) and boiling 3 min. Reactions were run on 8% SDS-PAGE and blotted to PVDF membrane (Hybond-P, Amersham) using a 3-solution semi-dry protein blotting protocol for 30 min at 16 V, optimized to a lower pH to avoid loss of phosphoester linkages.

Blotting setup in brief, from anode to cathode: one sheet of Wattman paper wet with Anode 1 solution (300 mM Tris pH 9.5, 10% (v/v) methanol), 2 sheets of Wattman paper wet in Anode 2 solution (25 mM Tris pH 9.5, 10% (v/v) methanol), PVDF membrane, gel, 3 Wattman sheets wet in Cathode solution (25 mM Tris pH 8.5, 20% (v/v) methanol, 0.3% (w/v) glycine). Phosphate incorporation was visualized by autoradiography.

Acid/Base Stability Assay

Autophosphorylation reactions were performed as above, in triplicate for each treatment. After blotting, PVDF membranes were incubated for 16h at room temperature in 1 M HCl, 3 M NaOH, or 100 mM Tris-HCl (pH 7.0). Proteins bands were cut from membrane and counted in scintillation fluid. The average for the counts of the acid and base treatments was normalized with respect to the counts for the neutral treatment (Tris-HCl).

Phosphoamino Acid Analysis

Autophosphorylation reactions were performed in 50 mM Tris pH 7.5, 10 mM MgCl2 (or MnCl2), 2 mM DTT, 10% (v/v) glycerol, 0.2 μM [γ-^{32}P] ATP (5000 Ci/mmol). After blotting, protein bands were cut from PVDF membranes and hydrolyzed in 100 μl 6 N HCl (Pierce) for 1h at 110^{o}C. Membrane was removed and hydrolyzed amino acids were lyophilized and resuspended in pH 1.9 buffer (2.2% (v/v) formic acid, 7.8% (v/v) acetic acid) containing 100 μg/ml each phosphoamino acid standard (Ser-P, Thr-P, Tyr-P (Sigma)). Bi-dimensional thin layer electrophoresis was performed as described in (Liu et al., 2002) and plates were visualized by autoradiography.

CHAPTER 3
STUDIES ON ETHYLENE RECEPTORS IN TOMATO

A family of six ethylene receptors have been identified from tomato: LeETR1, LeETR2 (Lashbrook et al., 1998), NEVERRIPE (NR) (Wilkinson et al., 1995), LeETR4, LeETR5 (Tieman and Klee, 1999), and LeETR6 (Tieman and Klee, unpublished). As shown in Figure 3-1, most of these receptors show the four domains defined for the Arabidopsis ETR1 protein, including a membrane spanning domain, a GAF domain, a kinase domain, and a receiver domain.

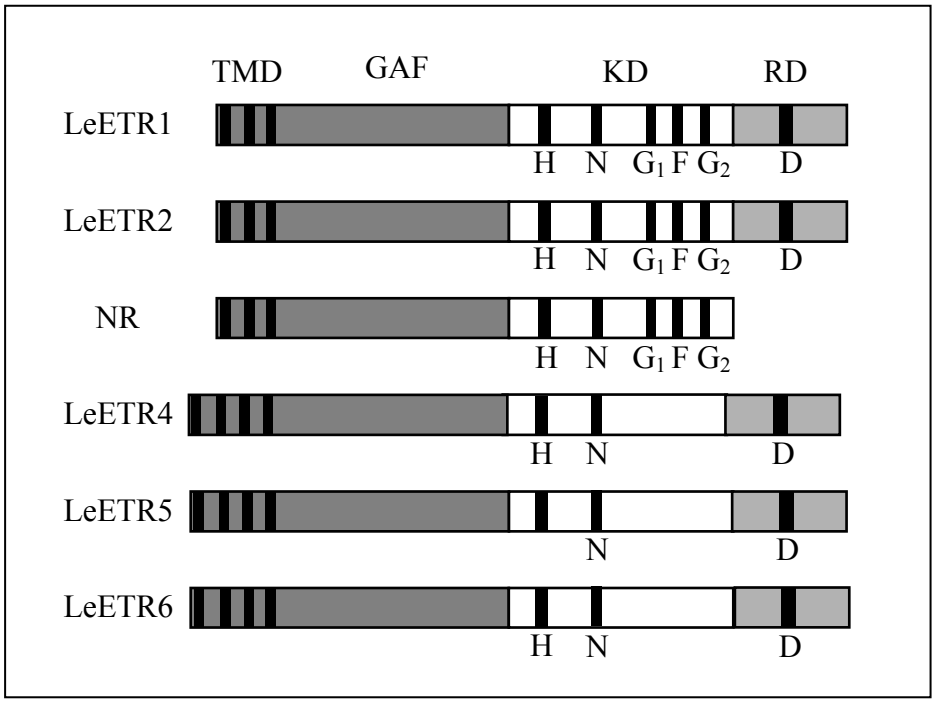

Figure 3-1. The tomato ethylene receptor family. The five conserved motifs necessary for histidine kinase activity (H-, N-, G1-, F-, and G2-box) are noted.

The most conserved domain is the trans-membrane (sensor) domain, which includes the regions required for dimerization and ethylene binding. The dominant insensitive *Nr* mutant has a point mutation in the sensor domain identical to the *etr2-1* mutation (Wilkinson et al., 1995; Sakai et al., 1998). The receiver domain is absent from NR, and LeETR5 does not contain the conserved histidine.

Comparative Studies on Gene Structure between Tomato and Arabidopsis

Similar to the Arabidopsis ethylene receptors, the tomato receptors can be divided into two subfamilies with respect to their sequence similarity. LeETR1, LeETR2 and NR are subfamily 1 receptors and have all the conserved motifs necessary for histidine kinase activity. The subfamily 2 class includes LeETR4, LeETR5 and LeETR6, which do not contain most of these motifs. The tomato subfamily 2 members also feature the putative fourth trans-membrane region at the amino-terminus (Figure 3-1). Figure 3-2 shows the phylogenetic relationship between the tomato and Arabidopsis ethylene receptors.

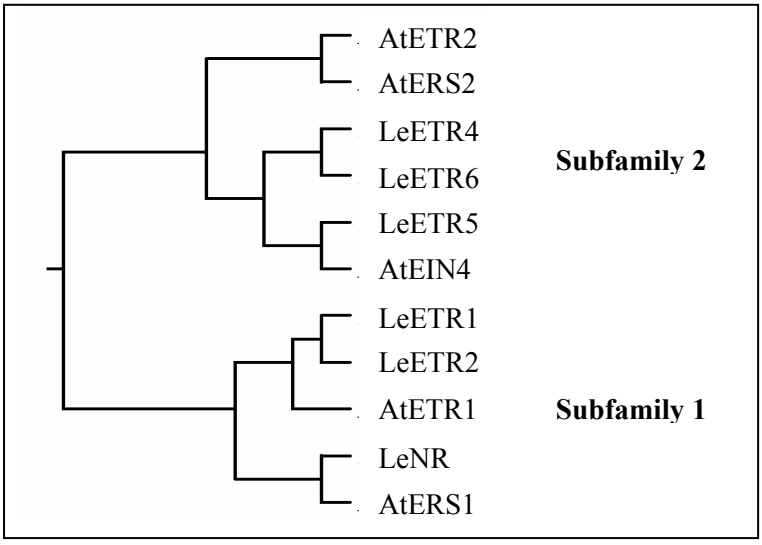

Figure 3-2. Sequence similarity tree of the Arabidopsis and tomato ethylene receptors. Neighbor-joining tree was generated from full-length protein sequences using Clustal W (http://clustalw.genome.ad.jp/).

Subfamily 2 divergences seem to have occurred after speciation, as no tomato orthologues to *AtETR2* and *AtERS2* or Arabidopsis orthologues *LeETR4* and *LeETR6* have been identified (Figure 3-2). The Arabidopsis subfamily segregation correlates not only with the conservation of kinase motifs, but also with the intron distribution of the genes in each family. As shown in Figure 3-3, *ETR1* has five introns in its coding sequence, one of which is in the receiver domain (Chang et al., 1993). *ERS1* has four introns in its sequence. When compared to *ETR1*, *ERS1* has introns at equivalent positions but lacks the receiver domain and its intron (Hua et al., 1995). *ETR2*, *ERS2* and *EIN4* have a single intron each at the same position in their sequence (Hua and Meyerowitz, 1998; Hua et al., 1998; Sakai et al., 1998). The Arabidopsis introns vary between 50 and 150 nucleotides (nt).

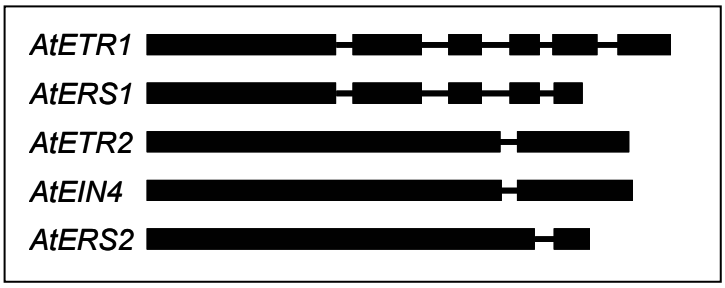

Figure 3-3. Gene structure for the Arabidopsis ethylene receptors *AtETR1*, *AtERS1*, *AtETR2*, *AtEIN4*, and *AtERS2*. Exons are shown as boxes and introns are shown as lines.

In order to determine the gene structure of the tomato receptors, the genomic sequence between the translational start and stop sites of the tomato ethylene receptors *LeETR2*, *Nr*, *LeETR4*, *LeETR5*, and *LeETR6* were isolated from *Lycopersicon esculentum*, as described in Chapter 2. The complete sequences of these genes are shown in Appendix A, and a graphic representation of their structure is shown in Figure 3-4. The tomato introns varied in size between 104 and 2863 nt, but were at conserved

positions when compared to members of their respective subfamilies. The *LeETR1* genomic sequence could not be cloned by the methods described in Chapter 2 because it had a 5000 nt intron approximately 80 nt after the ATG start codon and the PCR fragment was predicted to be over 10 kb (data not shown). The *LeETR1* start codon is 50 nt upstream when compared to the other subfamily 1 receptors and it is likely that the intron located in the 5' untranslated region (UTR) in the other subfamily 1 receptors is in the coding region of *LeETR1*. All Arabidopsis receptors have an intron in their 5' UTR (not shown) and the *NR* has two introns in its leader sequence, of 65 and 2400 nt (Figure 3-6).

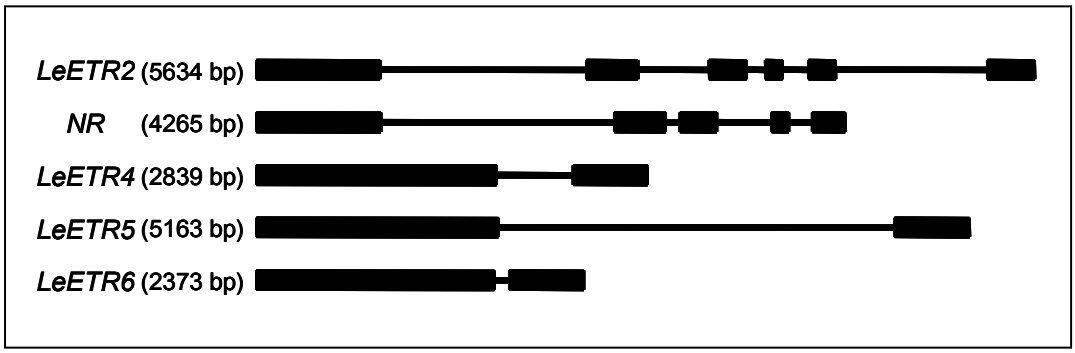

Figure 3-4. Gene structure for the tomato ethylene receptors *LeETR2*, *Nr*, *LeETR4*, *LeETR5*, and *LeETR6*. Exons are shown as boxes and introns are shown as lines.

Cellular Localization of EGFP fusions

The Arabidopsis ETR1 protein has been shown to localize to the endoplasmic reticulum (ER) by sucrose density-gradient centrifugation and immunoelectron microscopy (Chen et al., 2002). ETR1 has also been shown to be required for the recruiting of CTR1 to the ER membrane (Gao et al., 2003). In terms of ethylene signaling, this localization should not affect the recognition of the receptor by the hormone as ethylene is highly liposoluble. However, the subfamily 2 receptors have an

additional trans-membrane region at the amino-terminus, which could be a targeting sequence (Figure 3-1). TargetP (http://www.cbs.dtu.dk/services/TargetP-1.0/) was used to predict the subcellular localization of the tomato receptors (Emanuelsson et al., 2000). As shown in Table 3-1, TargetP predicted that the subfamily two receptors should be secreted, which suggests that this group localizes to the plasma membrane.

Table 3-1. Signal sequence prediction by TargetP.

Name	Location	Reliability Class	length of the presequence
LeETR1	-	2	-
LeETR2	-	4	-
NR	-	3	-
LeETR4	Secreted	1	21
LeETR5	Secreted	1	23
LeETR6	Secreted	2	23

*Reliability Class measures the size of the difference between the highest and the second highest output scores. There are five reliability classes; (1) represents the highest difference and (5) the lowest.

In order to address where the tomato receptors localize in the cell, the genomic sequences described above were cloned into an expression vector for localization studies. These constructs permitted the transient expression of the tomato receptors in tobacco protoplasts with the EGFP attached to their carboxyl-termini. The cDNAs were not used for expression because of their toxicity to bacteria, even when expressed at low levels. These constructs were sent to Dr. Mondher Bouzayen's laboratory at UMR 990, INRA-ENSAT- Genomique et biotechnologie des fruits, France, and the localization assays were done by Isabelle Milla. Our preliminary results suggest that the tomato receptors are also expressed in the ER. Figure 3-5 shows fluorescence images for a cell transfected with EGFP and a cell transfected with NR fused to EGFP. A Differential Interference Contrast (DIC) image was also acquired for each cell. Most of the protoplasts transfected with receptor fusions show fluorescence around the nucleus, which is consistant with ER

localization (Figure 3-5 and data not shown). As the labeling appears as dots (more or less expanded) around the nucleus, it seems unlikely that the receptors localize to the nuclear envelope. However, a marker that is specifically targeted to the ER is still needed to confirm these results.

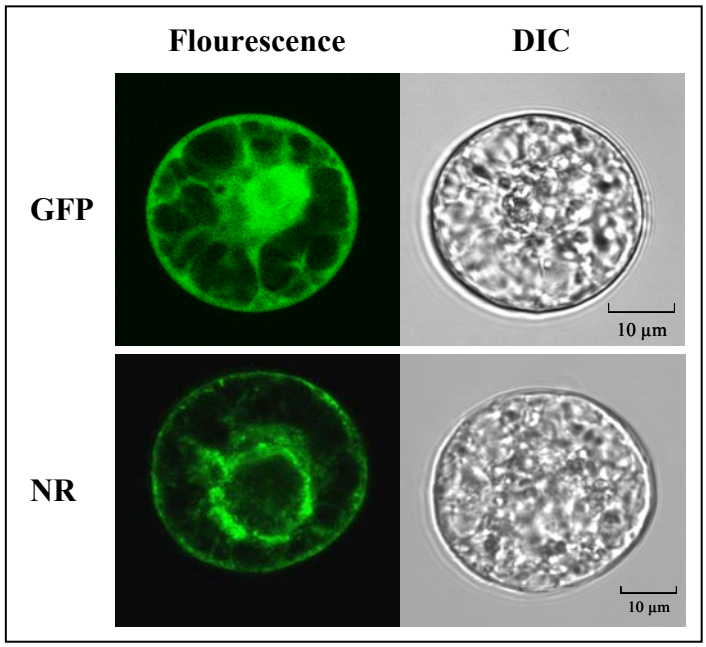

Figure 3-5. Cellular localization of the tomato receptors fused to EGFP. Transfected tobacco protoplasts were observed on a confocal laser scanning microscope. The samples were illuminated at 488 nm and the emission light collected between 500 and 525 nm. A Differential Interference Contrast (DIC) image was also acquired for each cell.

The Promoter Region of the *NR* Gene

The *NR* promoter was isolated from *L. esculentum* using the Genome Walker kit (BD Biosciences), as described in Chapter 2. Two kilobasepairs upstream of the putative transcription initiation site were isolated and sequenced. The transcription initiation site was determined by examining the longest cDNAs available for *NR*. The region between the site of transcriptional initiation and the start of the coding sequence was also isolated and sequenced in order to obtain the introns in this region. It has been previously

reported that the Arabidopsis receptors have a single intron in their 5' UTR (Chang et al., 1993; Hua et al., 1995; Hua and Meyerowitz, 1998; Sakai et al., 1998). The two introns identified in the *NR* leader sequence were 65 and 2400 nt long. A graphical representation of the *NR* locus is shown in Figure 3-6. It is also interesting to note that the 5' UTR of *NR* has several putative translational start sites, which create short open reading frames. These alternate initiation sites might be used to decrease translation of the protein and regulate receptor levels.

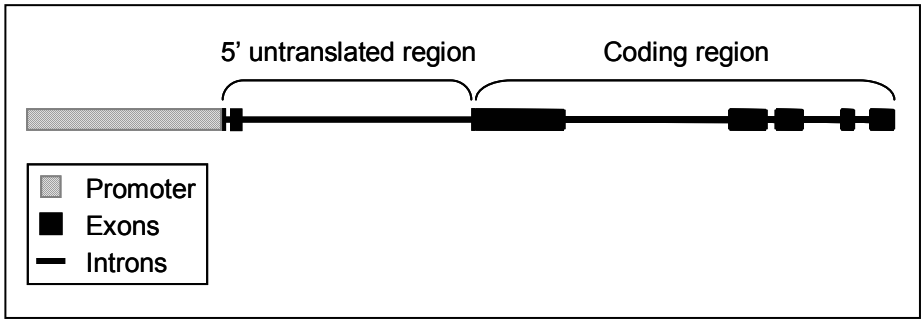

Figure 3-6. NR genomic locus.

The 4768 nt upstream of the start site of *NR*'s coding region are shown in Appendix B. The initiation site of transcription for *NR* has been predicted from cDNA clones to be around nucleotide 2015 of this sequence. PLACE Signal Scan Program (http://www.dna.affrc.go.jp/htdocs/PLACE/signalscan.html) was used to identify *cis*-acting elements in the promoter and leader sequences (Prestridge, 1991; Higo et al., 1999). Some of the *cis*-acting elements identified by the program are shown in Table 3-2 (identified by site number) and their location on the promoter sequence is shown in Appendix B.

Table 3-2. List of *cis*-acting elements identified by PLACE Signal Scan Program.

Site number	Putative Regulation Factor
s000024	auxin, SA, light
s000030	heat shock
s000124	light
s000142	pathogen response
s000148	seed
s000153	cold stress, ABA
s000167	dehydration, ABA
s000174	dehydration, ABA
s000175	dehydration, ABA
s000176	dehydration, ABA
s000177	dehydration, ABA
s000180	dehydration, ABA
s000181	dehydration, ABA
s000185	sucrose
s000198	SA, light
s000199	light
s000245	pollen
s000250	low temperature
s000252	circadian
s000256	sugar repression
s000259	sugar repression
s000263	ABA
s000264	seed
s000270	auxin
s000273	auxin, meristem, shoot, root, vascular tissue
s000292	ABA
s000298	GA
s000310	early defense responses
s000314	RAV1 binding site (VP1)
s000370	auxin
s000390	SA, disease resistance
s000392	light
s000401	ABA
s000403	sugar repression
s000407	cold stress, ABA
s000408	dehydration, ABA
s000409	dehydration, ABA
s000413	dehydration, ABA
s000414	etiolation
s000415	etiolation
s000421	seed
s000422	fruit
s000439	GA

*SA, salicylic acid; ABA, abscisic acid; GA, gibberellin.

The elements identified by PLACE Signal Scan seem consistent with published data for *NR* expression patterns and ethylene regulation. It has been previously shown that bacterial pathogen infection leads to increased expression of *NR* in tomato (Ciardi et al., 2000). Moreover, cross-talk between ethylene signaling and other phytohormones such as auxin and abscisic acid have been established at the physiological level but molecular data are still lacking (reviewed in (Davies, 1995; Ross and O'Neill, 2001).

Two programs were used to predict the TATA box of the *NR* promoter site (see Appendix B). PROSCAN version 1.7 (http://bimas.cit.nih.gov/molbio/proscan/) predicted a promoter region on the forward strand from nucleotide 456 to 706 and a TATA box was found at 670 (Prestridge, 1995). TSSP, a promoter prediction software for plant sequences (RegSite Plant DB, Softberry Inc., http://www.softberry.com), predicted three promoters in the given sequence with TATA boxes at nucleotides 303, 1341 and 1769. The site of transcription initiation for these promoters was predicted at nucleotide 326, 1375, and 1783, respectively. All these promoter predictions are too far from the start of transcription to be valid, which could be due to the limitations of the available programs. However, as the putative site of transcription initiation was determined by cDNA clones, it is possible that the leader sequence is actually longer than previously thought. It is possible that secondary structures in the messenger RNA have prevented longer cDNAs from being cloned. Determining the true site of transcription will facilitate the identification of the true promoter, and mutagenesis studies can be used to verify these predictions.

NR Promoter Expression Patterns by GUS fusions

The expression pattern of the *NR* gene has been studied using RNase protection assays (Lashbrook et al., 1998). This study showed that *NR* is regulated during tomato

fruit development: it is expressed at low levels in green fruit, its expression increases at the onset of ripening and declines after ripening has initiated. *NR* has also been shown to be expressed at low levels in seedlings, leaves, petals, ovaries, anthers, sepals, and abscission zones, while it shows a higher level in styles and petioles (Lashbrook et al., 1998). As shown in Figure 3-2, *ERS1* seems to be the Arabidopsis orthologue of *NR*. The expression of *ERS1* is also ubiquitous, but higher in young, small cells and reduced when cells are more expanded. *ERS1* is expressed in embryos, etiolated seedlings, leaves and stems; high expression is noticed in floral primordia and very strong expression is seen in anthers (Hua et al., 1995).

In this study we looked at *Nr* expression within tissues by attaching the *GUS* reporter gene to the *Nr* promoter. The promoter region described above was cloned into an *Agrobacterium* transformation vector and transformed into *L. esculentum* cv. *Micro-Tom*, as described in Chapter 2. Six independent transgenic lines were analyzed for *GUS* expression patterns by staining for GUS activity, as shown in Figure 3-7. *GUS* expression in these transgenic lines was seen in the anthers, style and stigma of the flower (A), vascular bundles of the stem (E) and mature seeds (I, J). GUS activity was low in immature fruit (F) and occurred predominantly in the calyx and columella. Expression increased during fruit maturation (G), was at its highest in ripening fruit (I) and decreased after the fruit was ripe (J). Mature seeds showed high levels of *GUS* expression, while immature seeds showed very little (K). GUS activity was also seen in flower buds, but this organ was also stained in wild-type plants. No expression was seen in leaves unless they were stained for three days (data not shown), with the exception of line 42, which showed expression in the leaf veins (D). This unique pattern of line 42 could be due to a

position effect, where the expression pattern is influenced by the factors surrounding the insertion site. However, *GUS* expression in the vascular bundles of the stem (E) was observed in multiple lines. The expression pattern observed in the *NR:GUS* transgenic lines correlate with the previous studies mentioned above (Hua et al., 1995; Lashbrook et al., 1998). These data suggest that GUS activity assays might be a useful technique to look at *NR* gene regulation and could be used to study *NR* expression patterns in response to different hormone treatments and biotic and abiotic stresses.

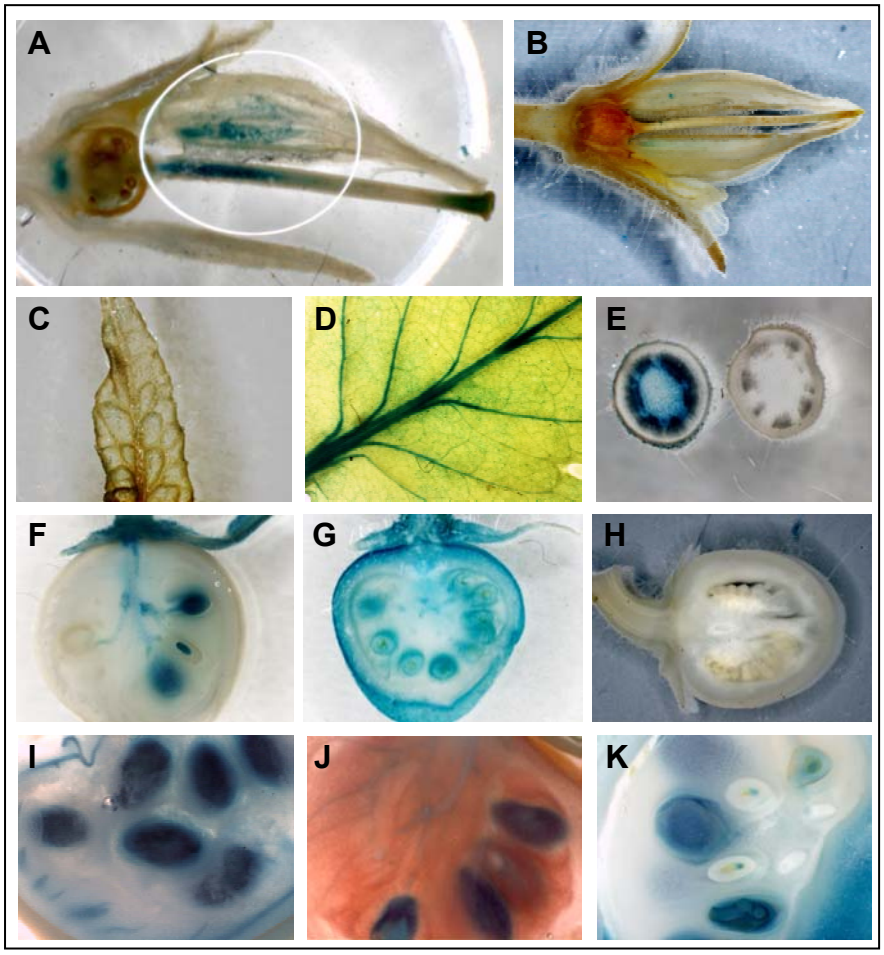

Figure 3-7. GUS activity in the *NR:GUS* transgenic lines. A, *NR:GUS* flower; B, wild-type (WT) flower; C, *NR:GUS* young leaf; D, *NR:GUS* old leaf; E, *NR:GUS* stem (left) and WT stem (right); F, *NR:GUS* immature fruit; G, *NR:GUS* mature green fruit; H, WT mature green fruit; I, *NR:GUS* ripening fruit; J, *NR:GUS* ripe fruit; K, *NR:GUS* mature and immature seeds.

CHAPTER 4
STUDIES ON RECEPTOR EXPRESSION LEVELS IN ARABIDOPSIS

One of the ways a plant can regulate hormone sensitivity is through the regulation of its receptors (reviewed in Weyers and Paterson, 2001; Gaspar et al., 2003). Plants can regulate the distribution pattern of ethylene receptors throughout the plant, as discussed in Chapter 3, and they can also regulate the amount of receptors expressed at a given time. Moreover, it has been suggested that the induction of receptors by ethylene is an alternative mechanism to limit the hormone response (Hall et al., 2000). Ethylene dissociation from the receptor does not appear to be a key regulator of response timing, as the half-life of ethylene binding is 12.5h (Schaller and Bleecker, 1995). Even though this measurement was taken from recombinant proteins expressed in yeast, it seems reasonable to predict that protein turnover removes ethylene-bound receptors, and *de novo* synthesis is used to replenish the receptor pool.

NR messenger RNA (mRNA) levels are regulated during tomato fruit development; its mRNA is at low levels in green fruit and it increases at the onset of ripening (Lashbrook et al., 1998). After ripening has initiated the levels of *NR* mRNA reduce somewhat, which correlates with the reduction of ethylene during ripening. *NR* mRNA levels are induced by ethylene and the mRNA levels during fruit ripening correlate with the level of ethylene produced by the fruit. *LeETR4* mRNA levels are also induced during fruit ripening, as well as during flower development (Tieman and Klee, 1999). Besides developmental regulation, ethylene receptor mRNA levels are also affected by

biotic and abiotic stresses. It has been previously shown that pathogen infection leads to increased mRNA levels of two ethylene receptor genes in tomato, *NR* and *LeETR4* (Ciardi et al., 2000). Furthermore, increases in *LeETR4* mRNA levels following infection is important in limiting the spread of necrosis (Ciardi et al., 2001). Abiotic stress such as flooding has also been shown to induce a *Rumex NR* homologue, *RpERS* (Vriezen et al., 1997).

A dramatic effect of transcriptional regulation of ethylene receptors has been observed in transgenic tomato plants. Antisense lines for *LeETR4* show a severe hypersensitivity to ethylene, while antisense lines for other ethylene receptors, such as *NR*, do not seem to show this phenotype (Tieman et al., 2000). The *LeETR4* antisense lines show increased leaf epinasty and premature flower abscission; fruit set in these lines requires treatment with ethylene inhibitors. Moreover, the lack of phenotype in the *NR* antisense lines is due to the higher levels of *LeETR4* mRNA in these lines, which compensates for the reduction in *NR* mRNA levels. Compensation for lack of *LeETR4*, however, does not occur in the *LeETR4* antisense lines. Thus, there is a differential regulation of gene expression of the tomato ethylene receptors. However, the ethylene hypersensitivity phenotype can be eliminated in *LeETR4* antisense lines by the overexpression of *NR*. Taken together these data support the hypothesis that the receptors perform redundant functions. Furthermore, they suggest that subfamily 2 receptors, such as LeETR4, are as capable of repressing the ethylene signal transduction pathway as the subfamily 1 receptor NR. These data also suggest that the receiver domain is not necessary for receptor signal transduction, as it is absent from the NR receptor (Tieman et al., 2000).

The large collection of Arabidopsis ethylene signaling mutants makes this species an excellent model for molecular biology studies of this signaling pathway. The goal of this study was to look at transcriptional regulation of the ethylene receptors in Arabidopsis by taking advantage of the existing mutant collection. All previous studies that looked at ethylene receptor mRNA levels in Arabidopsis were done by RNA blots. Due to the low expression levels of these receptors, RNA blots are not sensitive enough for comparative studies. More reliable information has been obtained from tomato expression studies using RNase protection assays and real-time RT-PCR (Lashbrook et al., 1998; Tieman and Klee, 1999; Tieman et al., 2001). Hence, real-time RT-PCR was used in this study in order to determine receptor mRNA levels in Arabidopsis, as described in Chapter 2. Receptor mRNA levels were measured in two different Arabidopsis ecotypes and the ability of ethylene to induce mRNA levels was investigated. Receptor mRNA levels were also determined for the ethylene insensitive mutant *etr1-1* and the constitutive ethylene response mutant *ctr1-10*. The effect of pathogen infection on receptor mRNA levels was also investigated, in order to look at the regulation of receptor gene expression due to biotic stress.

Receptor Expression in Arabidopsis

Several different ecotypes have been used in Arabidopsis studies in the past. The two ecotypes used in this study, Columbia and Wassilewskija (WS) have the same general morphology when grown under similar growth conditions. In order to determine if the receptor levels were equivalent in these two different ecotypes, receptor mRNA levels were measured in both Columbia and WS. Figure 4-1A shows that the major difference in receptor mRNA levels was seen in *ETR2*. *ETR2* mRNA is four-fold higher in Columbia than in WS. *ETR1* mRNA levels are higher in WS, while Columbia has

slightly higher levels of *ERS1*. The mRNA levels of *ERS2* and *EIN4* are the same in both ecotypes (Figure 4-1A).

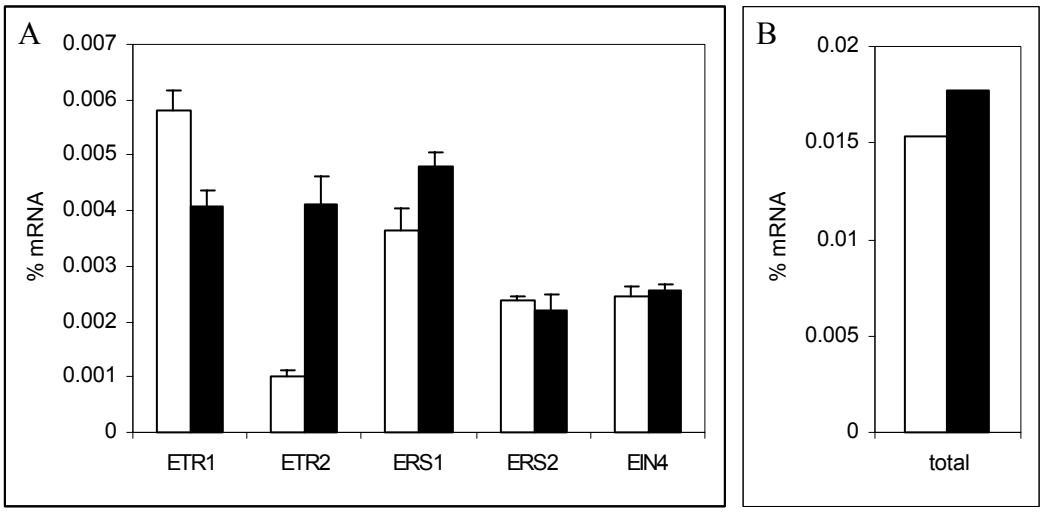

Figure 4-1. Receptor mRNA levels in rosette leaves of WS (white bars) and Columbia (black bars) ecotypes were determined by real-time RT-PCR using gene specific primers and TaqMan® probes to *ETR1*, *ETR2*, *ERS1*, *ERS2*, and *EIN4*. A, mRNA levels for individual receptors; B, total mRNA levels. Values expressed as percentage of mRNA ± SE (n=4).

Despite the differences observed in Figure 4-1A, there is not a significant change in the total mRNA levels of ethylene receptors between the backgrounds (Figure 4-1B). As no phenotypic or behavioral differences have yet been observed between these two ecotypes, these data support the hypothesis of receptor redundancy. Moreover, Figure 4-1A suggests that higher levels of the subfamily 1 receptor ETR1 can compensate for reduced levels of the subfamily 2 receptor ETR2. Taken together, these data seem to support the hypothesis that the two subfamilies have redundant roles; the total level of receptors determining the plant phenotype. It is important to note that these data correspond to steady-state mRNA levels and not protein quantifications. Moreover, a single biological sampling, despite comprising a pool of plants grown at the same time,

was used for these assays. Natural variation has not been taken into account and these results need to be verified with different pools of plants grown at the same time. Furthermore, it would be interesting to look at receptor mRNA levels in additional Arabidopsis ecotypes, in order to determine which receptors are expressed in the different backgrounds and if the total level of receptors is conserved.

Receptor Expression in Response to Ethylene

The induction of receptor expression levels by ethylene may be a mechanism to control the hormone response and delimit its window of action. Several physiological processes lead to the induction of ethylene, which is normally accompanied by an increase in mRNA levels of some receptors. *NR*, *LeETR4* and *LeETR5* are ethylene inducible tomato receptors, as determined by exogenous hormone treatments (Ciardi et al., 2000). However, fruit ripening only shows an increase of *NR* and *LeETR4* mRNA concomitant with the ethylene burst (Lashbrook et al., 1998; Tieman and Klee, 1999). Moreover, the plant's resistance response to pathogens leads to the increases in ethylene production and in *NR* and *LeETR4* mRNA levels (Ciardi et al., 2000). Therefore, even though *NR*, *LeETR4* and *LeETR5* are all ethylene inducible when the plant is treated with exogenous ethylene, other factors seem to be necessary for regulating receptor expression during physiological processes, as not all these receptors are induced in all instances.

In Arabidopsis, RNA blot studies using leaf tissue have suggested that *ETR2*, *ERS1*, *ERS2* showed a six-fold induction after 12 hours of exogenous ethylene treatment, while *ETR1* and *EIN4* were not ethylene inducible (Hua et al., 1998). As described in Chapter 2, a one hour exogenous ethylene treatment was used in this study, in order to look at early responses to exogenous ethylene. Due to the low mRNA levels of the ethylene receptors and the lack of sensitivity of RNA blots, mRNA levels were

determined for *ERS1*, *ETR2*, *ERS2*, and *EIN4* by real-time RT-PCR. Unfortunately, *ETR1* mRNAlevels are lacking for this experiment. As shown in Figure 4-2A, *ERS1* and *ERS2* are the only receptors that seem to be ethylene inducible under our assay conditions, and a two-fold induction was observed after a one hour treatment with ethylene. Moreover, after one hour there is already a 40% increase in the total mRNA levels in response to the ethylene treatment (Figure 4-2B), which could potentially be translated into an increase in receptor levels.

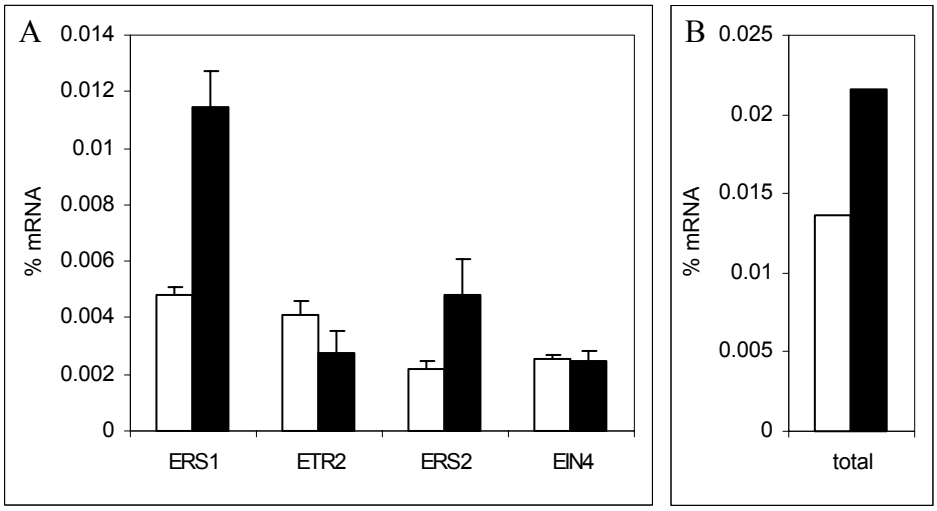

Figure 4-2. Effect of exogenous ethylene treatment on the mRNA levels of ethylene receptors in Columbia. A, mRNA levels for *ERS1*, *ETR2*, *ERS2*, and *EIN4* after air (white bars) or ethylene (black bars) treatment; B, total mRNA levels for air (white bars) or ethylene (black bars) treatments. Values expressed as percentage of mRNA ± SE (n=4). Total mRNA levels do not include *ETR1*.

These results suggest that *ERS1* and *ERS2* are rapidly induced in response to exogenous ethylene treatments, and could be involved in limiting the ethylene response. However, as mentioned above, only experimental error has been taken into account in these assays and biological replicates are needed to confirm these results. Moreover, it would be interesting to look at receptor mRNA levels at various time points during

treatments with exogenous ethylene, in order to determine the rate of *ERS1* and *ERS2* increase and when *ETR2* expression is induced.

Receptor Expression in Response to Pathogen Attack

Pathogen attack induces a complex and highly coordinated response from the plant. Immediate and long-term defenses are activated by local and systemic signaling mechanisms. There are two types of responses to pathogen infection, resistance and susceptibility. In a resistant response, the host actively inhibits pathogen growth and prevents the spread of disease. This response is dependent upon a specific host resistance (*R*) gene and a corresponding pathogen avirulence (*avr*) gene. *R* gene-mediated resistance is associated with rapid and localized cell death at the site of infection, termed the hypersensitive response (HR) (reviewed in (Yang et al., 1997).

In the absence of either the plant *R* or pathogen *avr* gene, a susceptible response occurs. In this case extensive disease development occurs and the virulent pathogen grows to a much higher titer. In the interaction between the bacterial pathogen *Xanthomonas campestris* pv. *campestris* (Xcc) and Arabidopsis the susceptible response is characterized by cell death at the infection site followed by spreading chlorosis and secondary necrosis in the surrounding uninfected tissue. However, susceptible hosts also possess a defense response that limits, but does not stop, pathogen growth. This phenomenon has been termed basal resistance. Although delayed in comparison to a resistant response, the susceptible response also leads to changes in gene expression. In both resistant and susceptible responses the host plant plays an active role in limiting pathogen growth and controlling disease symptom development (reviewed in (Glazebrook, 2001).

Despite the complexity of the host response to pathogens, a limited number of signaling intermediates are employed by the host to facilitate defense. In the case of resistance, three candidate defense hormones have been identified from diverse plant species infected with a variety of pathogens: salicylic acid (SA), ethylene, and jasmonic acid (JA). However, the specific plant-pathogen interaction determines which of these hormones are important (Yang et al., 1997). In tomato, both SA and ethylene are essential for development of disease symptoms in response to either *Xanthomonas campestris* pv. *vesicatoria* (Xcv) or *Pseudomonas syringae* pv. *tomato* (Pst) (O'Donnell et al., 2001). Removal of either of these hormones leads to tolerance, where pathogen growth is observed in the absence of disease symptoms. This indicates that although SA and ethylene are essential for symptoms they are not essential for basal resistance. Hormone analyses of mutant and transgenic lines indicated that ethylene-deficient plants do not produce SA following infection. Thus, alterations in ethylene responses directly affect SA levels and it is SA action that is associated with cell death (O'Donnell et al., 2001). In the compatible interaction between tomato and these bacterial pathogens, basal resistance is SA independent, whereas SA is essential for basal resistance in the interactions of Arabidopsis and a number of bacterial pathogens.

An ethylene response is observed in Arabidopsis infected with Xcc. In wild-type plants, there is an increase in ethylene synthesis at approximately 48 h after infection (O'Donnell et al., 2003). In the Arabidopsis-Xcc compatible interaction, preventing SA accumulation by expression of the *nahG* gene reduced subsequent ethylene production and altered the development of disease symptoms, with plants showing no visible chlorosis. However, ethylene insensitive lines, *etr1-1* and *etr2-1*, accumulated SA and

exhibited normal but precocious symptom development. Therefore, Arabidopsis, like tomato, was found to exhibit co-operative ethylene and SA action for the production of disease symptoms. However, in Arabidopsis, SA was found to act upstream of ethylene (O'Donnell et al., 2003).

It has been previously shown that pathogen infection leads to increased mRNA levels of two ethylene receptor genes in tomato, *NR* and *LeETR4* (Ciardi et al., 2001). This study also showed that the increased levels of *LeETR4* following infection is important in limiting the spread of cell death during the incompatible Xcv interaction. In order to determine whether regulation of receptor genes occurs in response to pathogen attack in Arabidopsis, steady-state mRNA levels of each of the ethylene receptors were quantified in infected tissue by quantitative real-time RT-PCR. As shown in Figure 4-3, three of the five ethylene receptors (*ETR1*, *EIN4* and *ERS2*) exhibited no alteration in mRNA levels following Xcc infection. Infection does, however, lead to alteration in the steady-state levels of *ETR2* and *ERS1* mRNA in Columbia. *ERS1* mRNA levels increased several-fold over time, but a greater effect of infection was seen on *ETR2* expression. Maximum levels were observed at 72 hpi concurrent with the peak in ethylene synthesis (O'Donnell et al., 2003). Measurement of receptor mRNA levels in the *etr1-1* and *etr2-1* mutants showed that ethylene action is required for the observed increase of *ETR2* and *ERS1* mRNA levels. This result is consistent with the observations described above that these two genes are ethylene-inducible (Figure 4-2A).

The increased *ETR2* and *ERS1* mRNA levels following infection may act to reduce ethylene sensitivity of the infected tissue. In tomato, Xcv infection leads to increased mRNA levels of *NR* and *LeETR4*, and increased receptor gene expression limits symptom

development by limiting ethylene action (Ciardi et al., 2000). Induction of expression of *ETR2* and *ERS1* was also absent from the NahG line (Figure 4-3). This effect may be a consequence of reduced ethylene in this line (O'Donnell et al., 2003). Since ethylene receptors are negative regulators of ethylene responses (Hua and Meyerowitz, 1998), the significant increase in receptor levels during infection would reduce overall ethylene sensitivity of infected tissue.

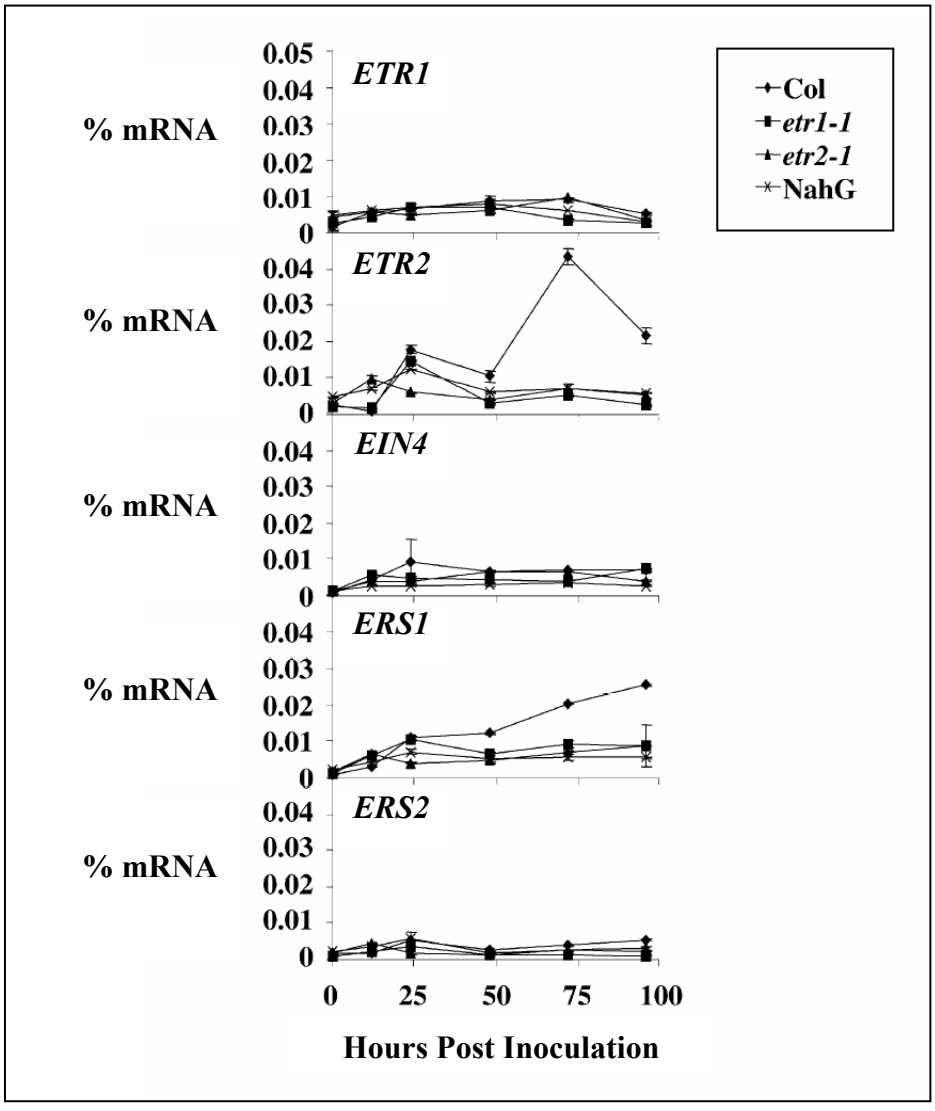

Figure 4-3. Receptor expression during pathogen response (adapted from O'Donnell *et al.*, 2003)

Receptor Expression in Arabidopsis Ethylene Signaling Mutants

Several mutants have been identified in the ethylene signal transduction pathway through triple response screens, including mutants insensitive to ethylene and mutants that signal in the absence of the hormone (Stepanova and Ecker, 2000). Here, mRNA levels were determined for *ETR1*, *ERS1*, *ETR2*, *ERS2*, and *EIN4* in the ethylene insensitive mutant *etr1-1* and the constitutive response mutant *ctr1-10*. The *etr1-1* mutation leads to ethylene insensitivity because this receptor can no longer bind the hormone (Hall et al., 1999). As ethylene binding releases the repressed state, the *etr1-1* mutant is constantly repressing the ethylene response. The *ctr1-10* mutant contains a loss-of-function allele of the *CTR1* gene, which encodes a Raf kinase-like protein (Kieber et al., 1993). CTR1 acts downstream of the ethylene receptors and is also a negative regulator of the ethylene response.

Previous work using RNA blot analysis showed that there are no differences in the transcription levels of *ETR1* and *etr1-1* (Chang et al., 1993; Zhao et al., 2002). Given the lack of sensitivity of RNA blots, real-time RT-PCR was used in this study, as described in Chapter 2. As shown in Figure 4-4A, *etr1-1* mRNA level is 30% less than *ETR1* levels in Columbia. However, as the previous expression *etr1-1* expression data were measured in seedlings (Zhao et al., 2002), it is not unreasonable to suppose that the mRNA levels of *etr1-1* might be decreasing through development, as the plant responds to the lack of an ethylene response. Moreover, the mRNA levels of *ERS1*, *ETR2*, and *ERS2* are lower in the *etr1-1* mutants when compared to wild-type Columbia (Figure 4-4B, C, D).

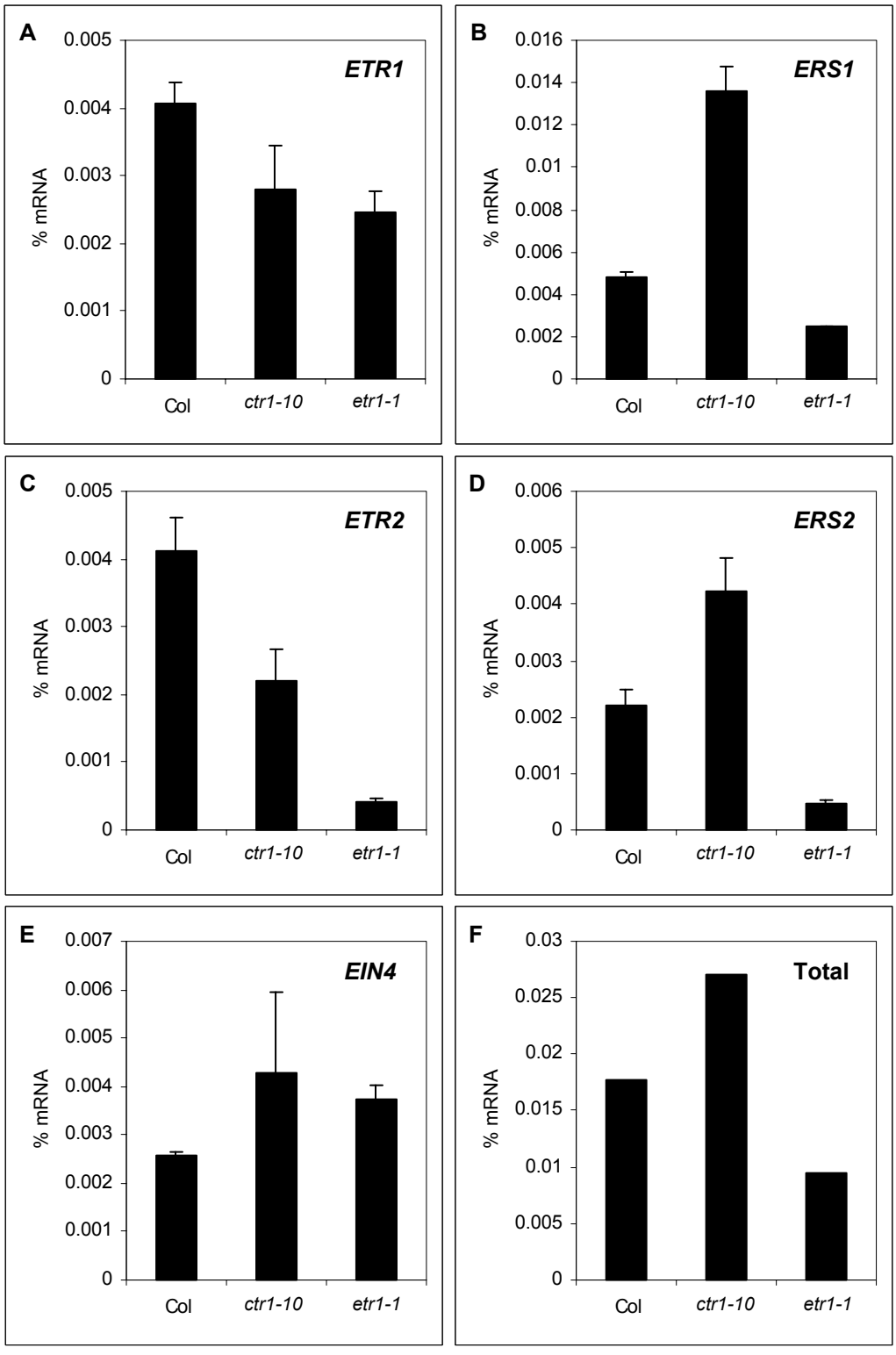

Figure 4-4. mRNA levels in the constitutive mutant *ctr1-10* and the insensitive mutant *etr1-1*. A, *ETR1*; B, *ERS1*; C, *ETR2*; D, *ERS2*; E, *EIN4*; F, total mRNA levels. Values expressed as percentage of messenger RNA ± SE (n=4).

The reduced level of total mRNA in the *etr1-1* mutant (Figure 4-4F) suggests that this plant is regulating the expression of its ethylene receptors in order to better respond to ethylene. This hypothesis is supported by the decrease of ETR1 protein in other insensitive lines, such as *etr2-1*, *ein4-1*, *ein2* and *ein3* (Zhao et al., 2002). Furthermore, regardless of the RNA levels of the *etr1-1* mutant, a two-fold increase in protein level has been observed in etiolated seedlings (Zhao et al., 2002). This higher protein level could be due to reduced turn-over of the mutant protein, or other mechanisms of post-translational regulation. However, these data suggest that the reduction in total receptor level (Figure 4-4F) might be the cause of ethylene insensitivity as the amount of mutant receptors might be greater than the wild-type ones. This hypothesis of a dosage effect is supported by data that show partial ethylene sensitivity in triploid *etr1-1* lines (Hall et al., 1999).

As shown in Figure 4-4 (B and D), the mRNA levels of *ERS1* and *ERS2* are higher in *ctr1-10* than in wild-type Columbia. The changes observed in the receptors' expression pattern in the *ctr1-10* mutant seem to correlate with the pattern observed after ethylene treatment (Figure 4-1). The total mRNA levels of receptor expression are increased in the *ctr1-10* mutant and as a response to exogenous ethylene treatment, suggesting that the ethylene effect observed in Figure 4-1 might be due to CTR1 inactivation by ethylene. Moreover, the mRNA levels of *ETR1* and *ETR2* were lower in the *ctr1-10* mutant, as shown in Figure 4-4 (A and C). However, Zhao *et al.* (2002) reported a two-fold increase in ETR1 protein levels in the *ctr1-2* mutant, which could suggest that a post-transcriptional regulation mechanism might be regulating ETR1. Taken together, these data suggest that the constitutive activation of the ethylene signal

transduction pathway seem to induce receptor expression in order to try to reduce the ethylene response. As the constitutive activation of the pathway is downstream of the receptor, these efforts are not effective and the signaling pathway remains activated.

As noted above, biological replicates are needed to confirm these results. Furthermore, it will be interesting to look at loss-of-function mutants of the ethylene receptors in order to see if their receptor expression pattern shows similarity to *ctr1-10*. No loss-of-function mutants for the ethylene receptors were identified in any of the large scale screens for ethylene response mutants, supporting the hypothesis that the receptor family members have redundant functions. Receptor loss-of-function mutants were obtained by either screens for intragenic suppressors of ethylene insensitive mutants or screens of T-DNA knockout populations (Hua and Meyerowitz, 1998; Zhao et al., 2002; Hall and Bleecker, 2003).

The single loss-of-function mutants have ethylene sensitivity kinetics similar to wild-type, but are shorter at any given point of the dose-response curve (Hua and Meyerowitz, 1998). Several crosses between these loss-of-function mutants have been made in order to create double, triple and quadruple loss-of-function mutants (Hua and Meyerowitz, 1998). Most double mutants do not appear to have a more severe phenotype than the single mutants, except the *etr1*;*ers1* double loss-of-function mutant (Hall and Bleecker, 2003). However, this could be due to the fact that *ETR1* and *ERS1* are the most highly expressed receptors in the WS background, which was used for the *ers1* mutant (Figure 4-1A). The triple loss-of-function mutants show several ethylene-inducible phenotypes when grown in air. These phenotypes are rescued by application of ethylene inhibitors, which suggests that the mutations only affect ethylene perception. These

mutants are slightly larger and healthier than the quadruple mutant, which has a very severe ethylene-sensitive phenotype (Hua and Meyerowitz, 1998). Preliminary studies using these loss-of-function mutant combinations have not shown any pattern of differential regulation of gene expression (data not shown). However, it was clear from the results obtained that biological replicates are essential for interpreting the data from these mutants.

CHAPTER 5
KINASE ACTIVITY OF THE ARABIDOPSIS ETHYLENE RECEPTORS

Two-component and phosphorelay signaling systems exist in both prokaryotes and eukaryotes. In the yeast *S. cerevisiae*, for example, there is only one histidine kinase sensor protein, the osmolarity receptor SLN1 (Ota and Varshavsky, 1993). SLN1 signals through a phosphorelay (Maeda et al., 1994; Maeda et al., 1995), which regulates the SSK2-PBS2-HOG1 MAP kinase cascade (Posas and Saito, 1998). In plants, there are several proteins that show sequence similarity to histidine kinases, including the phytochromes (Schneider-Poetsch et al., 1991) as well as hormone receptors for ethylene (Chang et al., 1993; Hua et al., 1995; Hua et al., 1998; Sakai et al., 1998) and cytokinins (Inoue et al., 2001). The cytokinin two-component signal transduction pathway has been suggested to function through a phosphorelay mechanism (Hwang and Sheen, 2001; Inoue et al., 2001).

ETR1 is an active histidine kinase, as it autophosphorylates a conserved histidine *in vitro* (Gamble et al., 1998). Like the yeast SLN1 signaling pathway, ethylene receptor signaling has been suggested to regulate the CTR1-SIMKK-SIMK/MMK3 MAP kinase cascade (Ouaked et al., 2003). However, due to the sequence divergence shown in Appendix C, it is doubtful that the subfamily 2 receptors can function as histidine kinases. Moreover, genetic data suggest that histidine autophosphorylation of ETR1 is not necessary for receptor function in ethylene signal transduction (Chang and Meyerowitz, 1995; Gamble et al., 2002; Wang et al., 2003). However, there is significant

evidence in the literature suggesting that histidine kinases can evolve into kinases that phosphorylate on serine residues. This phenomenon has been observed in the mitochondrial proteins branched chain α-ketoacid dehydrogenase (BCKD) kinase (Popov et al., 1992; Davie et al., 1995) and pyruvate dehydrogenase kinase (PDK) (Popov et al., 1993; Thelen et al., 2000), plant phytochromes (Yeh and Lagarias, 1998; Lapko et al., 1999), as well as a tobacco homologue of a subfamily 2 ethylene receptor (Xie et al., 2003). Hence, the goal of this study was to understand the kinase activity of the ethylene receptor family in Arabidopsis in order to provide insights into the mechanism of ethylene signal transduction.

Here, we show that all five Arabidopsis ethylene receptors autophosphorylate *in vitro*. However, ETR1 is the only family member that autophosphorylates exclusively on histidine residues. All other receptors show predominantly serine autophosphorylation under our assay conditions, and ERS1 autophosphorylates on both histidine and serine in the presence of Mn^{2+}. However, dual phosphorylation is not observed when ERS1 is assayed in the presence of Mg^{2+} and Mn^{2+}, suggesting that ERS1 might not have this activity *in vivo*. Moreover, mutation studies show that the histidine residue conserved in histidine kinases is not required for the serine autophosphorylation of the ethylene receptors. Hence, our results suggest that ethylene signal transduction in plants does not occur by a phosphorelay mechanism.

Expression of the Five Arabidopsis Ethylene Receptors in Yeast

In order to produce proteins suitable for *in vitro* enzyme assays, the soluble domains of the Arabidopsis ethylene receptors were cloned into pESP1 as described in Chapter 2. These constructs included the GAF domain, the kinase domain and the receiver domain, when the latter was present in the native protein, but lacked the amino-

terminal membrane-spanning domain. The soluble domains of all five ethylene receptors were expressed in *S. pombe*, each with a GST tag attached to its amino-terminus (Figure 5-1). A 70 kDa protein co-purified with most ethylene receptors and could not be removed even after extensive washes. Sequencing of the ETR2 70 kDa contaminating band determined that the co-purifying protein was the heat shock protein Hsp70 (data not shown). The molecular chaperone Hsp70 is usually removed by addition of Mg^{2+} and ATP to the column washes (Sherman and Goldberg, 1991), but such a treatment was not possible in this case as it could interfere with the *in vitro* autophosphorylation activity of the ethylene receptors. As Hsp70 has ATPase activity but has not been shown to have kinase activity, hence it was not removed from the reaction mixture.

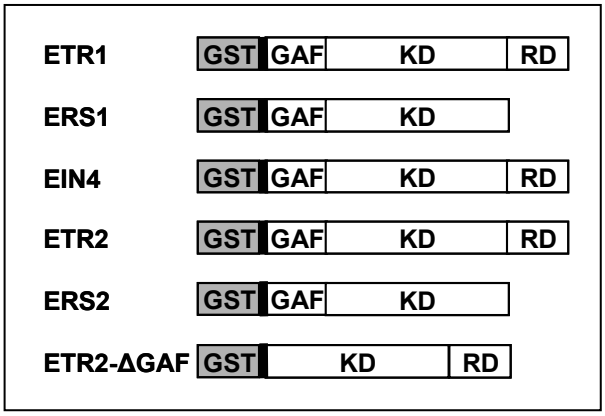

Figure 5-1: Ethylene receptor constructs expressed in yeast. Soluble domains of the Arabidopsis receptors were cloned into a yeast expression vector as described in Chapter 2 and expressed as GST fusions. The thrombin cleavage site (bold line) was used for GST removal from the fusion protein. ETR1, ERS1, ETR2, EIN4, and ERS2 included the GAF domain along with the kinase domain (KD) and receiver domain (RD), when present in the native protein. A construct was also made for ETR2 that deleted the GAF domain (ETR2-ΔGAF).

Autophosphorylation Activity *in vitro*

Purified recombinant receptors were tested for autophosphorylation *in vitro* as described in Chapter 2, and results are shown in Figure 5-2.

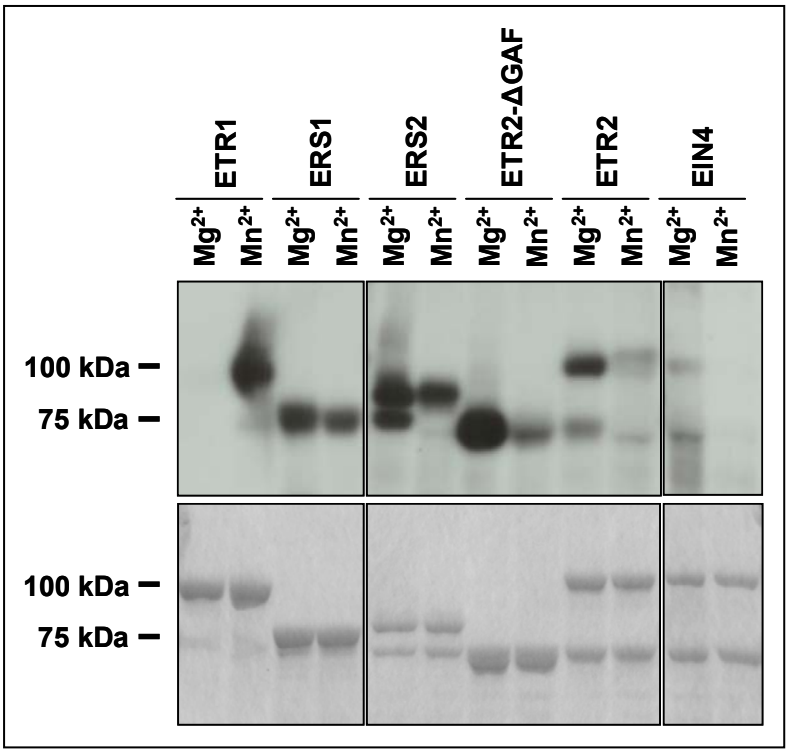

Figure 5-2: *In vitro* autophosphorylation activity and cation dependence. Ethylene receptors were tested for autophosphorylation *in vitro* in the presence of Mg^{2+} and Mn^{2+} as described in Chapter 2. Autoradiogram of the protein blot (top) and a stained gel of the proteins used (bottom) are shown for ETR1 (95 kDa), ERS1 (81 kDa), ERS2 (81 kDa), ETR2-ΔGAF (73 kDa), ETR2 (95 kDa), and EIN4 (96 kDa).

As previously reported (Gamble et al., 1998), ETR1 required Mn^{2+} for autophosphorylation and did not function in the presence of Mg^{2+}. ERS1 and ERS2 autophosphorylated in the presence of Mg^{2+} or Mn^{2+}, while ETR2 and EIN4 had a higher activity in the presence of Mg^{2+}. The recombinant EIN4 protein was very unstable and could not be purified in large quantities. Several independent clones were tested for EIN4 with similar results in the autophosphorylation assay (data not shown). It is interesting to note that ERS2, ETR2 and EIN4 were able to phosphorylate Hsp70 in the presence of Mg^{2+}. There are other kinases that have their activity differentially regulated by Mg^{2+} and Mn^{2+}. An example is the p21-activated protein kinase γ-PAK, which has

higher autophosphorylation activity in the presence of Mn^{2+} but only phosphorylates its substrate in the presence of Mg^{2+} (Tuazon et al., 1998). Moreover, Hsp70 phosphorylation by the ethylene receptors might not be altogether circumstantial as Hsp70 has been shown to interact with receptors and kinases to activate stress responses in eukaryotes (reviewed in Nollen and Morimoto, 2002)).

As shown in Figure 5-3, GST alone showed no phosphorylation, indicating that phosphorylation is dependent on the ethylene receptors being present in the reaction mixture. Moreover, phosphorylation was also observed when ERS1 was purified without the GST tag (ERS1-GST(-)), indicating that the site of phosphorylation is internal to the receptor. The reduced autophosphorylation of the ERS1-GST(-) in the presence of Mn^{2+} might be due to the instability of the recombinant protein after the thrombin digestion.

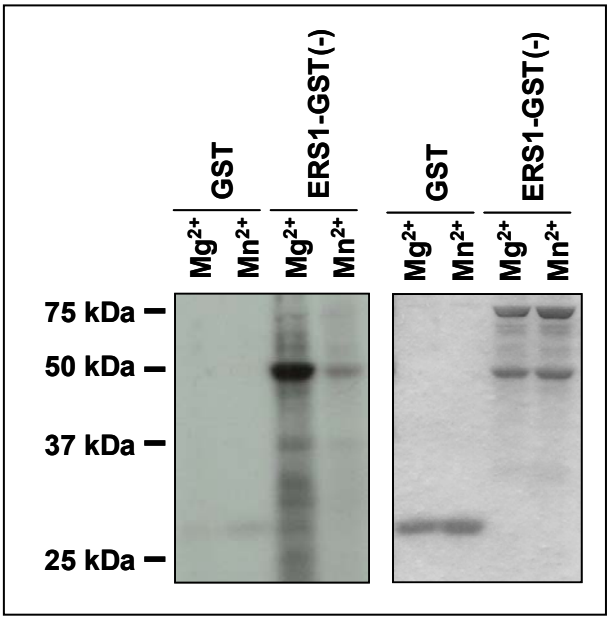

Figure 5-3: *In vitro* autophosphorylation activity and cation dependence. GST (28 kDa) and ERS1 without the GST tag (ERS1-GST(-); 55 kDa) were tested for autophosphorylation *in vitro* in the presence of Mg^{2+} and Mn^{2+} as described in Chapter 2. Autoradiogram of the protein blot (left) and a stained gel of the proteins used (right) are shown.

We also tested whether the GAF domain is required for autophosphorylation by using an ETR2 construct lacking this domain (ETR2-ΔGAF; Figure 5-1). As shown in Figure 5-2, ETR2-ΔGAF showed the same autophosphorylation pattern as the full-length ETR2 construct. This result also suggests that the GAF domain is not the site of autophosphorylation. Hence, the phosphorylated residue must reside in the kinase domain as ERS1 and ERS2 do not contain receiver domains (Hua et al., 1995; Hua et al., 1998).

Nature of the Phosphorylated Amino Acid

In order to determine the nature of the phosphorylated amino acid, autophosphorylated proteins were incubated in acid or base as described in Chapter 2. Phosphorylated histidine residues form phosphoamidate bonds that are sensitive to acid and resistant to base, while phosphorylations on serine, threonine, and tyrosine produce phosphoester bonds that are acid-resistant and base-labile. Moreover, aspartate phosphorylation is labile in both acid and base. As has been previously reported (Gamble et al., 1998), autophosphorylation of ETR1 in the presence of Mn^{2+} resulted in a base stable phosphorylated residue under our assay conditions, consistent with histidine autophosphorylation (Figure 5-4). Low levels of incorporation were quantified from ETR1 reactions containing Mg^{2+}. The phosphorylated residue of CDPKα shows acid stability, consistent with its serine and threonine autophosphorylation (Putnam-Evans et al., 1990).

As shown in Figure 5-4, ERS1, ETR2, EIN4, and ERS2 showed acid stability in the presence of Mg^{2+}, indicating a phosphoester bond formation. In the presence of Mn^{2+}, ERS1 showed partial resistance to both acid and base, suggesting that this protein has dual activity and can produce phosphoamidate and phosphoester linkages in the presence

of this metal. The subfamily 2 class of ethylene receptors only produced phosphoester linkages, independent of the metal present in the reaction mixture (Figure 5-4). Low levels of incorporation were quantified from ETR2 reactions containing Mn^{2+}, but ETR2-ΔGAF in the same buffer showed enough incorporation for quantification (Figure 5-4).

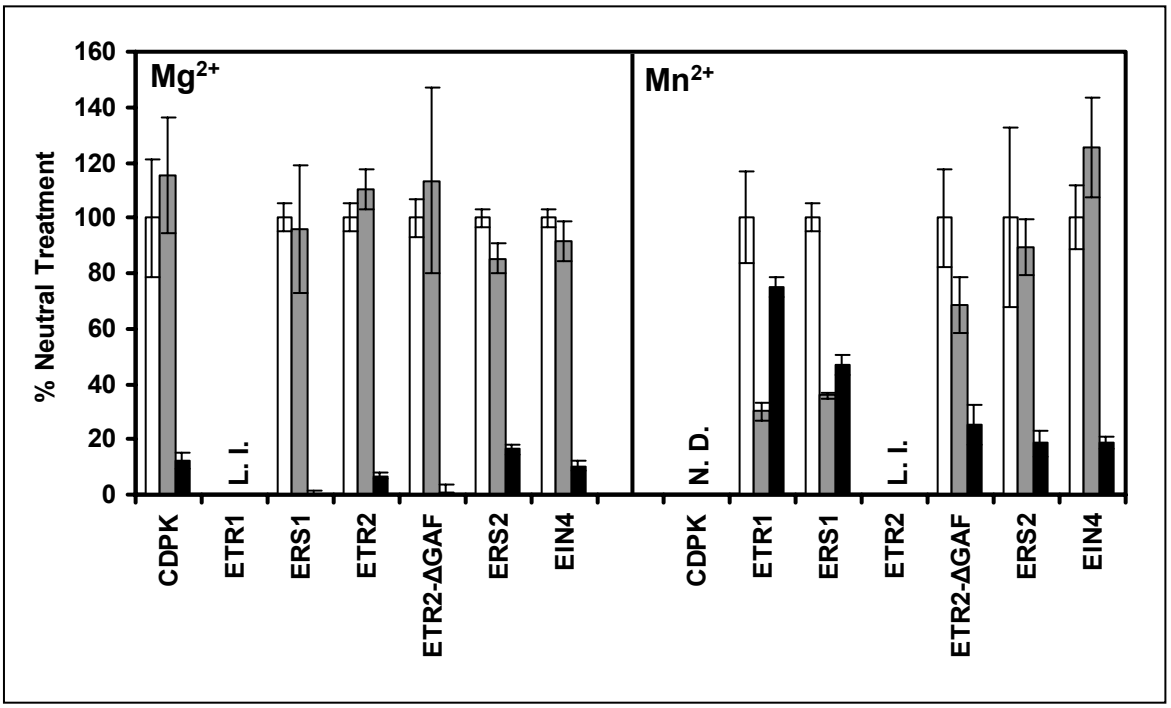

Figure 5-4: Acid and base stability of phosphorylated amino acids. Autophosphorylation reactions were performed as described in Chapter 2, in triplicate for each treatment, in a total of nine reactions for each protein. Reaction products were run on SDS-PAGE, and blotted to PVDF membranes. Membranes were incubated for 16h in neutral (white bars), acidic (gray bars) or basic (black bars) solutions before individual protein bands were cut from membranes and counted in a scintillation counter. Graphs show the average of three values for each treatment (± SE), normalized to the counts for the neutral treatment. L. I., low incorporation; N. D., not determined.

Bi-dimensional thin layer electrophoresis was used to determine whether the phosphoester bond was formed on serines, threonines, tyrosines, or combinations thereof and results are shown in Figure 5-5. ERS1 only autophosphorylated on serine residues in the presence of Mg^{2+}. All receptors were tested in the presence of Mg^{2+} or Mn^{2+} and all

autophosphorylated predominantly on serine residues (data not shown). ETR1 did not show any significant phosphorylation on serine, threonine or tyrosine in the presence of Mn^{2+} (Figure 5-5) and Hsp70 phosphorylation also occurred predominantly on serine residues (data not shown). Faint traces of threonine phosphorylation were only observed for ERS2 in the presence of Mn^{2+} (Figure 5-5) and for Hsp70 phosphorylated by EIN4 and ERS2 in the presence of Mn^{2+} (data not shown).

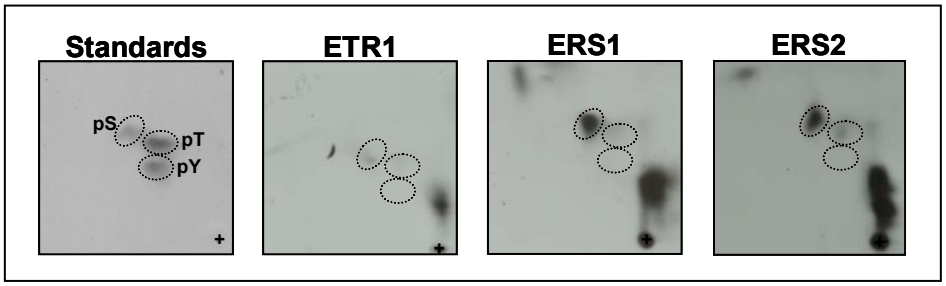

Figure 5-5: Phosphoamino acid analysis of autophosphorylated receptors. Autophosphorylation reactions were performed as described in Chapter 2, run on SDS-PAGE and blotted to PVDF membrane. Protein bands were cut from PVDF membranes, hydrolyzed in HCl and subjected to two-dimensional thin-layer electrophoresis. The autoradiograms of the plates are shown for ETR1 (Mn^{2+}), ERS1 (Mg^{2+}) and ERS2 (Mn^{2+}), and the positions of the standard phosphorylated serine, threonine, and tyrosine are marked.

In order to address the biological relevance of the different autophosphorylated sites of ERS1 we tested for autophosphorylation in the presence of both Mg^{2+} and Mn^{2+}. As the cellular concentration of free Mg^{2+} is 50 to 100-fold higher than Mn^{2+} (reviewed in Mukhopadhyay and Sharma, 1991), the autophosphorylation reaction was performed taking this ratio into account. Under the conditions used for the autophosphorylation reaction the calculated apparent concentrations of free Mg^{2+} and Mn^{2+} are 9.5 mM and 0.14 mM, respectively, as described in Chapter 2. As shown in Figure 5-6, in the presence of both metals, ERS1 only showed serine phosphorylation, suggesting that the

histidine phosphorylation of ERS1 probably does not occur *in vivo*, unless a Mn^{2+} donor is present.

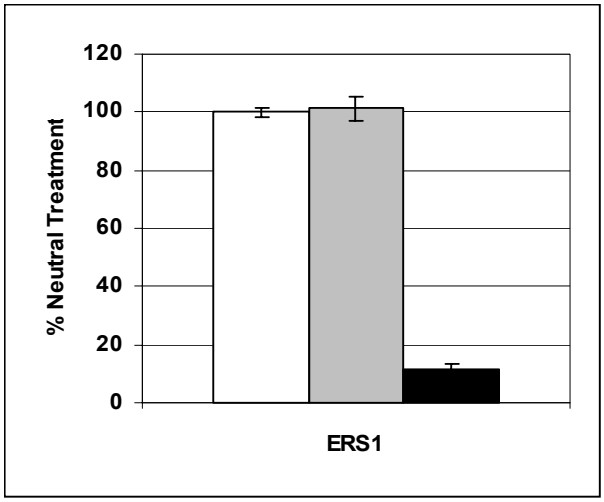

Figure 5-6: ERS1 autophosphorylation in the presence of both Mg^{2+} and Mn^{2+}. ERS1 was tested for autophosphorylation *in vitro* in the presence of both Mg^{2+} and Mn^{2+} as described in Chapter 2. Reactions were performed in triplicate for each treatment. Reaction products were subjected to SDS-PAGE, and blotted to PVDF membranes. Membranes were incubated for 16h in neutral (white bar), acidic (gray bar) or basic (black bar) solutions before individual protein bands were cut from membranes and counted in a scintillation counter. Values shown ± SE.

Insights on the Mechanism of Phosphorylation

Since the ethylene receptors are ancestral histidine kinases, it is possible that the observed serine phosphorylation occurs through an intramolecular transfer from a phosphorylated histidine, although this phenomenon has not been previously observed. Three of the five receptors contain the conserved histidine, while four of the five contain a histidine residue extremely close to the H-box (Appendix A). The neighboring histidine is not phosphorylated in ETR1 (Gamble et al., 1998). To examine whether the conserved and neighboring histidine residues are required for the autophosphorylation of the ethylene receptors, we made constructs that changed these histidine residues to

alanines, as shown in Figure 5-7. The ETR2-ΔGAF deletion construct was used for this study as it behaved like the full length ETR2 protein. The ERS2 protein sequence shows no histidine residue in this region (see Appendix A), so it was not used in this study.

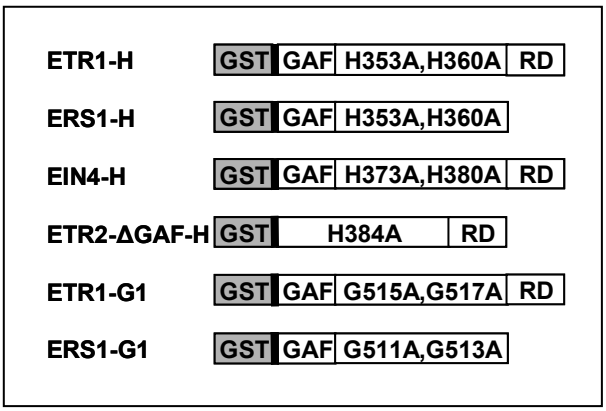

Figure 5-7: Ethylene receptor mutants expressed in yeast. ETR1, ERS1, ETR2-GAF and EIN4 were mutated as described in Chapter 2 and expressed as GST fusions. Mutated proteins lack the conserved and/or neighboring histidine of the H-box or the two glycines of the G1-box.

As previously reported (Gamble et al., 1998), ETR1-H did not autophosphorylate in the presence of Mg^{2+} or Mn^{2+} (Figure 5-8). Consistent with the dual activity of ERS1, a reduction of autophosphorylation activity was observed for ERS1-H in the presence of Mn^{2+}. The mutations in ERS1-H, ETR2-ΔGAF-H, and EIN4-H did not abolish autophosphorylation, as occurred with ETR1-H. Hence, it is improbable that serine phosphorylation of the ethylene receptors is due to an intramolecular phosphoryl transfer. These data indicate that the histidine residue of the H-box is not essential for autophosphorylation on serine residues and that no phosphoryl transfer is occurring from histidine to serine in the ethylene receptors.

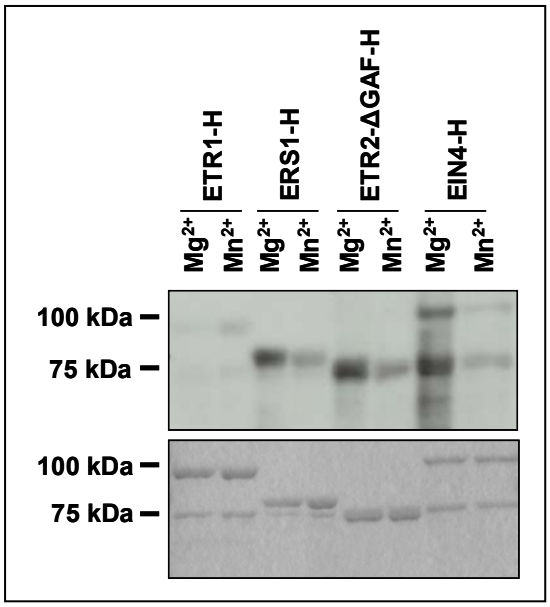

Figure 5-8: Effects of histidine mutations on *in vitro* autophosphorylation activity. The conserved histidine and/or the secondary histidine of ETR1 (95 kDa), ERS1 (81 kDa), ETR2-ΔGAF (73 kDa), and EIN4 (96 kDa) were mutated as described in Chapter 2. The expressed proteins were then assayed for *in vitro* autophosphorylation activity in the presence of Mg^{2+} or Mn^{2+}. Both the autoradiogram of the protein blot (top) and a stained gel of the proteins used (bottom) are shown.

The ATP binding domain of histidine kinases follows the Bergerat fold, which is conserved in enzymes with different functions such as the chaperone Hsp90, the DNA mismatch repair enzyme MutL and type II DNA topoisomerases (Dutta and Inouye, 2000; Koretke et al., 2000). The primary sequence similarity between these enzymes is less than 15%, but their secondary structure is conserved (Dutta and Inouye, 2000). As the ethylene receptors seem to be ancestral histidine kinases, it is likely that the mechanism of ATP binding should be conserved in these proteins, whether they phosphorylate on serines or histidines. In order to test whether ATP binding occurs by the same mechanism, we mutated the G1-box of ERS1. The G1-box is a glycine-rich loop region that is involved in ATP binding (reviewed in (Stock et al., 2000). According

to Gamble *et al.* (1998), the G1-box mutation completely abolishes histidine phosphorylation in ETR1. As shown in Appendix A, ETR1 and ERS1 are the only ethylene receptors with all the recognizable motifs that seem to be necessary for histidine kinase activity (Parkinson and Kofoid, 1992). The subfamily 2 class of receptors does not have the conserved residues of the G1-box motif and was not used for this study. However, as shown in Figure 5-9, mutation of the G1-box did not abolish autophosphorylation activity of ERS1 or ETR1 under our assay conditions.

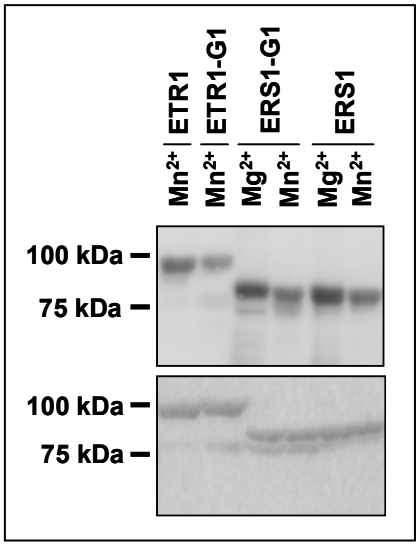

Figure 5-9. Effects of G1-box mutations on *in vitro* autophosphorylation activity of ETR1 and ERS1. The conserved glycines of ETR1 (95 kDa) and ERS1 (81 kDa) were mutated as described in Chapter 2. The ETR1 mutant protein (ETR1-G1) was assayed for *in vitro* autophosphorylation activity in the presence of Mn^{2+}, along with wild-type ETR1. The ERS1 mutant (ERS1-G1) was assayed in the presence of Mg^{2+} or Mn^{2+}, along with wild-type ERS1. Both the autoradiogram of the protein blot (top) and a stained gel of the proteins used (bottom) are shown.

The ETR1-G1 and ERS1-G1 plasmids were sequenced from the yeast clones to make sure the G1 box was mutated. The data in Figure 5-9 contradict the previously published results that the G1 box is essential for ETR1 kinase activity (Gamble et al., 1998) and put into question whether the mechanism of ATP binding is the same for

histidine kinases and ethylene receptors. It will be interesting to know if the ethylene receptors have a conserved Bergerat structure even though the amino acids of the designated boxes are not conserved, but protein structure data are necessary to address these issues.

CHAPTER 6
CONCLUSIONS AND FUTURE DIRECTIONS

Ethylene is one of the most studied phytohormones and it has several roles in plant development. It is involved in leaf and flower senescence, defense responses, fruit ripening, leaf and fruit abscission, and seed germination (Abeles et al., 1992). Several components of the ethylene signal transduction pathway have been identified in the last two decades. Five proteins (ETR1, ERS1, ETR2, ERS2, and EIN4) have been identified in Arabidopsis as receptors for ethylene (Chang et al., 1993; Hua et al., 1995; Hua et al., 1998; Sakai et al., 1998). These receptors show four distinct domains: a membrane spanning domain, which contains the ethylene binding site (Schaller and Bleecker, 1995); a GAF domain (Aravind and Ponting, 1997); a kinase domain with sequence similarity to histidine kinases (Parkinson and Kofoid, 1992); and a receiver domain as found in response regulator proteins (Stock et al., 2000). The receiver domain, however, is absent from ERS1 and ERS2 (Hua et al., 1995; Hua et al., 1998). Six ethylene receptor family members have been identified in tomato: LeETR1, LeETR2 (Lashbrook et al., 1998), NR (Wilkinson et al., 1995), LeETR4, LeETR5 (Tieman and Klee, 1999), and LeETR6 (Tieman and Klee, unpublished). These receptors show the four domains defined for the Arabidopsis ETR1 protein, as discussed in Chapter 3.

The most conserved domain in all ethylene receptors is the trans-membrane (sensor) domain, including the amino acids that are required for dimerization and ethylene binding. The ethylene receptor family can be further divided into two

subfamilies. Subfamily 1 receptors have all the conserved motifs necessary for histidine kinase activity, while the subfamily 2 members do not. Some subfamily 2 members even lack the conserved histidine that is phosphorylated in two-component and phosphorelay systems. This subfamily segregation does not correspond to the presence or absence of the receiver domain, but does correlate with intron distribution within the genes, as shown in Chapter 3. The subfamily 2 members also feature a putative fourth trans-membrane region at the amino-terminus consisting of 20-30 hydrophobic amino acids, which could be targeting sequences as discussed in Chapter 3. The Arabidopsis ETR1 protein has been shown to localize to the ER (Chen et al., 2002), which allows for the recruiting of CTR1 to the ER membrane (Gao et al., 2003). In terms of ethylene signaling, this localization should not affect the recognition of the receptor by the hormone as ethylene is highly liposoluble. As shown in Chapter 3, the tomato ethylene receptors seem to localize to the ER, irrespective of their subfamily.

In Arabidopsis, *in situ* hybridization studies show that *ETR1* is expressed ubiquitously, at low levels in seedlings and higher levels in stems and flowers. *ETR1* is strongly expressed in anthers and carpels throughout development (Hua et al., 1998). The expression of *ERS1* is ubiquitous; it is expressed in embryos, etiolated seedlings, leaves and stems. High levels of *ERS1* expression are noticed in floral primordia and very strong expression is seen in anthers (Hua et al., 1998). *In situ* hybridization studies showed that *ETR2* is evenly, but weakly, expressed in all tissues, with higher levels in the central inflorescence meristem and in young floral meristems. In flowers, *ETR2* expression is higher in developing ovules and petals (Sakai et al., 1998). *EIN4* is expressed in several tissues as shown by RT-PCR and RNA blots; *in situ* studies showed

that the expression level is low and ubiquitous, but stronger in stamens. The expression pattern of *ERS2* is the same as for *EIN4*, both genes being expressed in etiolated seedlings, leaves, roots, stems, and inflorescences. High expression is seen in stamens and ovules. In later floral development *ERS2* expression was localized to epidermal layers of the septum, where the other four genes were not expressed (Hua et al., 1998). Taken together these data suggest that ethylene receptors are expressed in all tissues throughout development, even though not all receptors are present in all tissues at all times.

The *NR:GUS* transgenic lines described in Chapter 2 were used to look at the expression pattern of *NR* within tissues. In these transgenic lines GUS activity was seen in the anthers, style and stigma of the flower, vascular bundles of the stem and mature seeds. *GUS* expression was low in immature fruit and predominantly located in the calyx and columella. Expression increased during fruit maturation and spread throughout the fruit. It was highest in ripening fruit and decreased when the fruit was ripe. Mature seeds showed high levels of *GUS* expression, while immature seeds showed very little. The expression pattern observed in the *NR:GUS* transgenic lines correlates with the previous studies on *NR* expression (Hua et al., 1995; Lashbrook et al., 1998) and the data presented in Chapter 3 suggest that GUS activity assays might be a useful technique to study *NR* expression patterns in response to different hormone treatments and stresses.

Plants can regulate the distribution pattern of ethylene receptors in different tissues and they can also regulate the levels of receptors expressed at a given time. A dramatic effect of transcriptional regulation of ethylene receptors has been observed in *NR* antisense plants, where *LeETR4* expression is increased to compensate for the lack of *NR*

(Tieman et al., 2000). As discussed in Chapter 4, one of the goals of this study was to look at transcriptional regulation of the ethylene receptors in Arabidopsis. The two ecotypes used in this study, Columbia and Wassilewskija have the same general morphology when grown under similar growth conditions. However, some differences in receptor mRNA levels were observed in these ecotypes, primarily higher levels of *ETR2* mRNA were found in Columbia. Despite the differences observed when comparing the steady-state RNA levels of individual genes, there was not a significant change in the total level of ethylene receptors between the ecotypes. The data presented in Chapter 4 are consistent with the hypothesis that the two subfamilies have redundant roles and that the total level of receptors determines the plant's phenotype.

As discussed in Chapter 4, *ERS1* and *ERS2* seem to be induced immediately after the exogenous ethylene treatment. After one hour there was already a 40% increase in the total mRNA level in response to the treatment. Moreover, it has been reported that *ETR2*, *ERS1*, *ERS2* showed a six-fold induction after 12 hours of exogenous ethylene treatment, while *ETR1* and *EIN4* were not ethylene inducible (Hua et al., 1998). No increase in *ETR2* mRNA level was seen after one hour ethylene treatment, under our assay conditions. As discussed in Chapter 4, *ETR2* is expressed during pathogenesis in an ethylene-dependent manner. Hence it seems likely that this gene should be ethylene inducible. The lack of visible *ETR2* expression after one hour of exogenous ethylene treatment could be due to *ETR2* being induced later than *ERS1* and *ERS2* or having a slower rate of induction that prevented its detection after only one hour. A more detailed study is needed to establish the expression pattern of these receptors after exogenous ethylene treatment. It would be interesting to look at receptor expression levels at

various time points during treatments, in order to determine the rate of receptor induction. This more thorough analysis would help determine if and when *ETR2* expression is induced in response to ethylene treatment.

The pathogen response data presented in Chapter 4 have recently been published (O'Donnell et al., 2003). Arabidopsis infection with the bacterial pathogen Xcc leads to increases in mRNA levels of *ETR2* and *ERS1*. *ERS1* mRNA levels increased several-fold over time, but a greater effect of infection was seen on *ETR2* mRNA levels. Maximum levels were observed after 72 hours, which was concurrent with the peak in ethylene synthesis. Measurement of receptor mRNA levels in the ethylene insensitive mutants *etr1-1* and *etr2-1* showed that ethylene perception is required for the observed increase of *ETR2* and *ERS1* mRNA levels. Moreover, this result is consistent with the observations described in Chapter 4 that these two genes are ethylene-inducible in wild-type plants. However, no *ERS2* induction was observed after pathogen infection, suggesting that the pathogen might be interfering with the ethylene response.

One of the advantages of studying ethylene response in Arabidopsis is the large mutant collection available for this signaling pathway. As discussed in Chapter 4, receptor mRNA levels were determined in *etr1-1* and the constitutive ethylene response mutant *ctr1-10*. Receptor mRNA levels were reduced in the former and increased in the latter. The reduced level of total mRNA in the *etr1-1* mutant suggests that this plant is decreasing the levels of its ethylene receptors to better respond to ethylene. The opposite is true for the *ctr1-10* mutant, where mRNA levels increase in an apparent attempt to block the constitutive ethylene response. The changes observed in the receptors' mRNA levels in the *ctr1-10* mutant seem to correlate with the patterns observed after ethylene

treatment. As the receptors are negative regulators of the ethylene signal transduction pathway, it will be interesting to look at receptor expression in loss-of-function mutants of the ethylene receptors to see if their expression patterns show similarity to *ctr1-10*. Single loss-of-function mutants do not show a constitutive ethylene response phenotype (Hua and Meyerowitz, 1998), which could be due to compensation by other ethylene receptors to maintain a constant level of receptors. This phenomenon has been observed in tomato transgenic lines between *LeETR4* and *NR* (Tieman et al., 2000) and could also occur in Arabidopsis.

The results presented in Chapter 3 and 4 support the hypothesis that the receptors are redundant in function. However, the sequence divergence between these receptors calls into question whether they have the same biochemical function. The Arabidopsis ETR1 protein is an active histidine kinase, as it autophosphorylates a conserved histidine *in vitro* (Gamble et al., 1998). However, the subfamily 2 receptors do not contain the conserved motifs that seem to be necessary for histidine kinase activity (Appendix C). Moreover, genetic data suggest that histidine autophosphorylation of ETR1 is not necessary for receptor function in ethylene signal transduction (Chang and Meyerowitz, 1995; Gamble et al., 2002; Wang et al., 2003). As shown in Chapter 5, all five Arabidopsis ethylene receptors autophosphorylate *in vitro*, independent of the presence or absence of the histidine kinase conserved motifs. While these results corroborate the previously published histidine autophosphorylation activity of ETR1 (Gamble et al., 1998), the other four members of the ethylene receptor family autophosphorylate predominantly on serine residues. As receptor kinase activity was maintained despite the

sequence divergence, it seems reasonable to predict that autophosphorylation should be important for receptor function.

There are several examples of serine autophosphorylation by proteins with sequence similarity to histidine kinases, including the plant phytochromes (Yeh and Lagarias, 1998; Lapko et al., 1999) as well as the mitochondrial proteins BCKD kinase (Davie et al., 1995) and PDK (Thelen et al., 2000). Figure 6-1 shows a sequence similarity tree comparing ethylene receptors to known histidine kinases and to kinases that phosphorylate on serine residues despite their similarity to histidine kinases. The subfamily 1 of ethylene receptors are in a clade with cytokinin receptors CRE1 and CKI1 (Hwang and Sheen, 2001; Inoue et al., 2001) and other histidine kinase homologues from Arabidopsis (Urao et al., 2001). The kinase domains of Arabidopsis and tomato ethylene receptors are closely related to the kinase domain of eukaryotic phytochromes, but not to the kinase related domain present in these proteins. The latter seems to have arisen from a duplication of the kinase domain and does not have *in vitro* kinase activity. The kinase related domain of plant phytochromes is in a separate clade along with PDK, BCKD kinase and the *E. coli* histidine kinase CheA.

The canonical histidine kinases SLN1, EnvZ and Cph1 (cyanobacterial phytochrome) are not in a clade with either of the groups of serine-phosphorylating enzymes, and sequence similarity between these groups is less than 15%. The phylogenetic relationship between these canonical and non-canonical histidine kinases suggests that the ability of histidine kinases to phosphorylate on serine residues evolved independently multiple times. Furthermore, the ethylene receptor family seems to be showing degrees in this evolution from histidine to serine phosphorylation. ETR1 and

ERS1 have all the histidine kinase motifs, but ETR1 autophosphorylates on histidines while ERS1 has histidine and serine phosphorylation activity. At the other end of the spectrum there is ERS2, which has none of the histidine kinase motifs and phosphorylates on serines and threonines.

Phytochromes, BCKD kinase and PDK have maintained the conserved amino acids of the ATP-binding domain (Popov et al., 1992; Popov et al., 1993; Yeh and Lagarias, 1998) while the ethylene receptor subfamily 2 members have not. Hence, it is probable that these specific amino acids are not required for serine autophosphorylation. Moreover, the phosphorylation mechanism used by BCKD kinase is different from the one used by canonical histidine kinases. Instead of attacking the γ-phosphate of ATP with the side chain of the phosphate-accepting histidine in the H-box (Bilwes et al., 1999) it uses a glutamate in the N-box as a general base catalyst to activate the serine to be phosphorylated (Tuganova et al., 2001). The ethylene receptors possess conserved glutamates and aspartates in close proximity to the N-box (Appendix C) that could be used by the enzyme to catalyze the phosphate transfer. Moreover, the results presented in Chapter 5 suggest that the conserved histidine residue of the H-box is not required for serine autophosphorylation of the ethylene receptors, as its exchange to an alanine residue does not abolish autophosphorylation. However, a definite answer to whether the ATP binding domain is conserved in the subfamily 2 receptors will require resolving the structure of their kinase domains. Structure data will also help identify amino acids that might be involved in the phosphorylation mechanism.

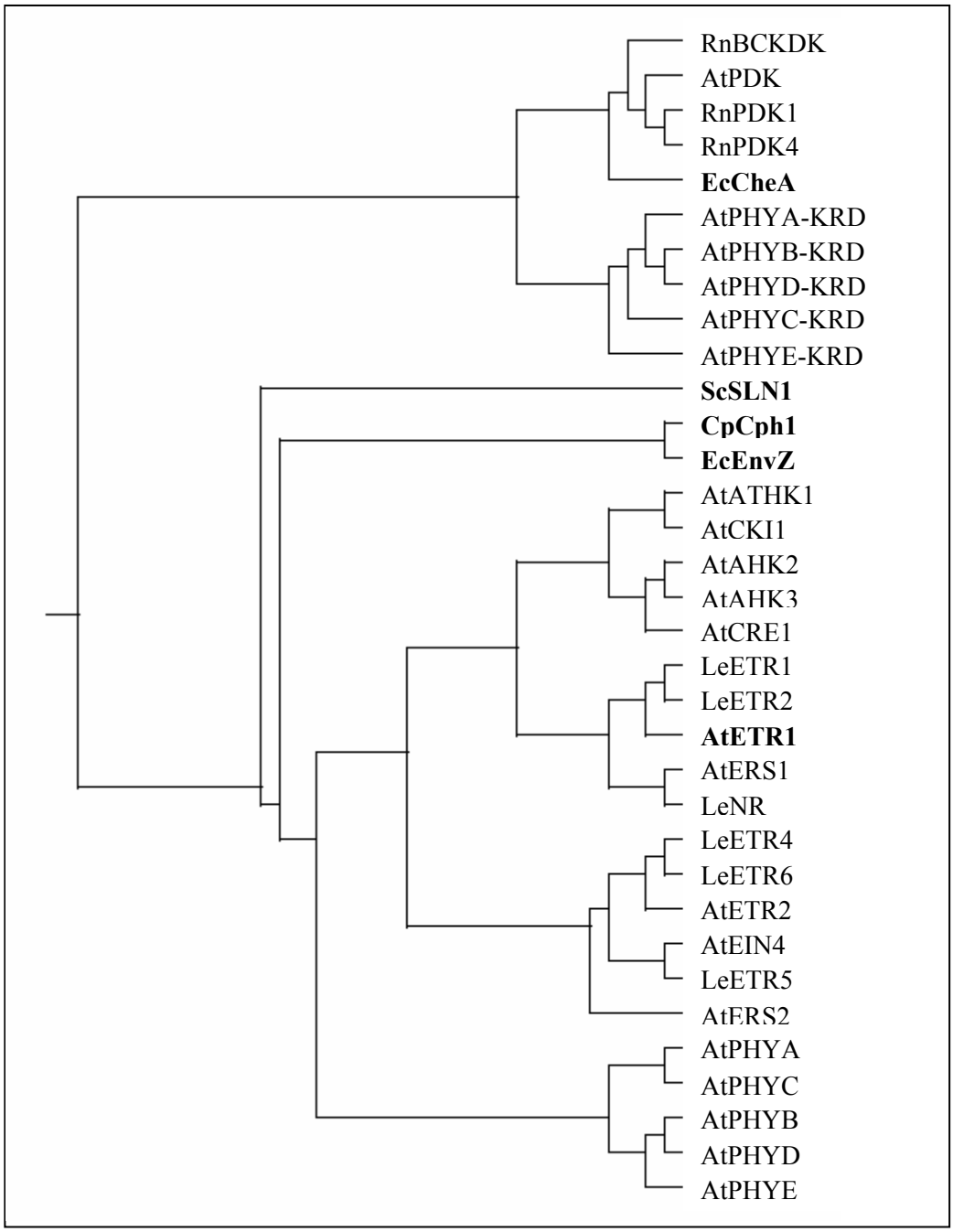

Figure 6-1. Phylogenetic relationship between the kinase domains of Arabidopsis and tomato ethylene receptors, phytochromes (PHY), the mitochondrial proteins PDK and BCKDK, the cytokinin receptors CRE1 and CKI1, histidine kinase homologues in Arabidopsis (ATHK1, AHK2, AHK3), and canonical histidine kinases (bold) SLN1, CheA, EnvZ and Cph1. Neighbor-joining tree was generated from full-length protein sequences using Clustal W (http://clustalw.genome.ad.jp/). KRD, kinase related domain present in eukaryotic phytochromes.

The proposed mechanism for histidine kinase phosphorylation suggests that trans-phosphorylation occurs between subunits of homodimers (reviewed in Stock et al., 2000). Hence, it can be inferred that canonical and non-canonical histidine kinases should be able to phosphorylate other proteins. This phenomenon has been observed for phytochromes (Ahmad et al., 1998; Fankhauser et al., 1999), BCKD kinase (Popov et al., 1992; Davie et al., 1995), and PDK (Popov et al., 1993; Thelen et al., 2000), and could be true for the ethylene receptors. No CTR1 phosphorylation by ethylene receptors has been shown to date, even though these proteins interact *in vitro* and *in vivo* (Clark et al., 1998; Gao et al., 2003; Huang et al., 2003). It is possible that the ethylene receptors could phosphorylate another component of the signal transduction pathway that has not been identified. As mentioned in Chapter 5, the ethylene receptors show substrate phosphorylation activity *in vitro*, as they are able to phosphorylate Hsp70.

The biochemical data presented in Chapter 5 show that the five Arabidopsis ethylene receptors show kinase activity *in vitro*, but do not phosphorylate on identical amino acids. These data, however, do not completely disagree with the proposed functional redundancy of the receptors. Genetic and biochemical data suggest that all family members are active in ethylene signal transduction (Hua and Meyerowitz, 1998; Hall et al., 1999), but it is not clear whether kinase activity is the primary means by which the receptors signal. It has been reported that loss of histidine autophosphorylation or removal of the kinase domain of ETR1 does not impair ethylene insensitivity conferred by the dominant insensitive *etr1* mutant (Gamble et al., 2002). Neither do mutations that disrupt histidine kinase activity of ETR1 prevent its complementation of *etr1;ers1* double loss-of-function mutants (Wang et al., 2003). Hence, it has been

suggested that receptor kinase activity is not part of the mechanisms of ethylene signal transduction.

This conclusion, however, is based solely on data for ETR1 and histidine kinase activity. Moreover, it relies on the assumption that the receptor's kinase is active in the absence of ethylene and responsible for the "ON" state. It has been demonstrated that CTR1 kinase activity is required for the repression of ethylene signaling (Huang et al., 2003), yet the *in vivo* kinase activity of the ethylene receptors has not been studied. Histidine kinase activity of ETR1 is not necessary for the repression of the ethylene signal transduction pathway (Gamble et al., 2002; Wang et al., 2003), but if autophosphorylation is involved in protein turnover, for example, it would not be necessary for the maintenance of the repressed state. The receptors could be modulating CTR1 kinase activity directly by a change in their conformation in response to ethylene binding; autophosphorylation of the receptors could be responsible for CTR1 turnover after ethylene binding. It seems unlikely that phosphorylation is required for CTR1 inactivation, as ETR1 mutants that abolish phosphorylation do not lead to ethylene insensitivity (Gamble et al., 2002). This model is consistent with lack of receptors inducing ethylene response in the absence of ethylene (Hua and Meyerowitz, 1998) as it would prevent CTR1 from localizing to the ER membrane where signaling is occurring (Gao et al., 2003).

It cannot be ruled out that the receptors might be sequestering a downstream component of the signaling pathway that is released upon ethylene binding and protein phosphorylation. In this scenario, phosphorylation could lead to receptor turnover or a change in its conformation, either of which would lead to release of the sequestered

component. In order to answer these questions it will be necessary to know if serine phosphorylation occurs *in vivo* and whether serine phosphorylation is required to maintain the repressed state or to release this repression. The biochemical data presented in Chapter 5 provide support for the genetic evidence that histidine autophosphorylation is not necessary for maintaining the repressed state (Chang and Meyerowitz, 1995; Gamble et al., 2002; Wang et al., 2003), and suggest that receptor signaling in the "ON" state does not occur through a phosphorelay. However, as kinase activity has been retained despite the sequence divergence of the ethylene receptor family, it seems likely that this activity is important for receptor function.

Regardless of the differences in enzymatic activity of the ethylene receptors, their functional redundancy still seems to be a valid hypothesis. Several observations contributed to this hypothesis, including the identification of dominant insensitive alleles for all the receptor family members (Bleecker et al., 1988; Hua et al., 1995; Hua et al., 1998; Sakai et al., 1998), the observation that the two subfamilies can compensate for each other in tomato (Tieman et al., 2000) and the inability to identify loss-of-function mutants in genetic screens. Moreover, single loss-of-function mutations of these receptors do not lead to a constitutive ethylene response (Hua and Meyerowitz, 1998), which further supports the hypothesis that the receptor family members might have redundant functions. Triple and quadruple loss-of-function mutants have more pronounced phenotypes that mimic a constitutive ethylene response (Hua and Meyerowitz, 1998). The only data inconsistent with the redundancy hypothesis is the observation that the subfamily 2 receptors are not able to complement the *etr1;ers1* double loss-of-function mutant, which shows a severe constitutive ethylene phenotype

(Hall and Bleecker, 2003). However, it has been suggested that the subfamily 2 receptors do not bind CTR1 as tightly as the subfamily 1 receptors (Cancel and Larsen, 2002). This difference in affinity for CTR1 could account for the lack of complementation observed with the *etr1;ers1* double mutant. Nevertheless, a more detailed biochemical study of the receptors and their effect on CTR1 function is needed in order to determine the mechanism of ethylene signal transduction.

APPENDIX A
TOMATO GENOMIC SEQUENCES

LeETR2

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  51  GAAGTATCAG TACATTTCTG ATTTTTTCAT TGCAGTTGCT TATTTTTCCA
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 201  ACACCTTATC AATTTGTGGA CTTCTACTCC TCATACAAGG ACTGTGGCAA
 251  TGGTGATGAC TACGGCGAAG TTCTCCACTG CTGCGGTATC ATGTGCAACT
 301  GCTGTCATGC TTGTGCACAT TATTCCGGAT TTATTAAGTG TCAAAACTAG
 351  GGAGCTATTC TTGAAAAACA AAGCGGCGGA ACTTGATCGT GAAATGGGTC
 401  TTATTCGGAC ACAGGAGGAG ACGGGTAGAT ATGTTAGAAT GCTAACACAT
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 701  TTCGACCTAC CCGGAAGTAC ATTCCAGGTG AGGTGGTTGC TGTTAGGGTC
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2501 GTAACAAGTA AGCTACTTGA AAAATTGGGA TGCAGTGTTT CTGCAGTTTC

2551 ATCTGGACGT GACTGTATTG GTGTTCTTAG CCCTGCTGTA TCCTCATTCC

2601 AAATCGTCCT TTTGGATCTT CACCTGCCTG ATTTGGATGG CTTCGAAGTA

2651 ACCATGAGAA TTCGGAAGTT TGGTAGCCAC AACTGGCCAT TGATAGTTGG

2701 TTTAACTGCG ACTGCTGATG AAAATGTTAC TGGAAGATGC CTGCAGATTG

2751 GAATGAATGG ACTTATTCGT AAACCAGTGC TATTGCCAGG TATCGCTGAT

2801 GAGCTTCAAA GGGTTCTGCT ACGGGGAAGT AGAATGATGT AA

LeETR5

1 ATGTTGGCAA TGTTAAGGTT GTTGTTTCTG GTACTGTTGA TTTCTTTGGT

51 CATTATATCT GTTTCAGCTA ATGATGGTGA ATTCTTCAAT TGCTGTGATG

101 AAGATGGTTT TTGGAGTATA CATACTATTT TAGACTGCCA GAAAGTGAGT

151 GACTTCTTTA TTGCTGTTGC TTACTTTTCT ATCCCTCTTG AGTTGCTTTA

201 CTTCATTAGC CGCTCGAATC TTCCTTTCAA ATGGGTTCTA GTTCAGTTCA

251 TTGCTTTTAT AGTGCTTTGT GGATTGACAC ATTTGCTCAA TGGATGGACT

301 TACAATCCTC ATCCTTCTTT CCAATTGATA TTGTCCCTAA CCGTTGCGAA

351 AATCCTTACT GCCCTTGTAT CTTGTGCAAC TGCAATCACC CTTTTGACTC

401 TGATCCCTCT TCTCCTAAAG ATAAAGGTCA GAGAACTTTT TTTGGCACAG

451 AATGTTTTAG AGCTAGATCA AGAGGTTGGG ATGATGAAGA AACAAACAGA

501 AGCTAGCATG CATGTCCGTA TGTTGACACA CGAAATTAGG AAGTCACTTG

551 ATAAGCACAC AATATTATAC ACTACACTAG TTGAACTTTC GAAAACCTTA

601 AAGTTGCAGA ATTGTGCTGT TTGGATGCCA AATGAAAGTA GGTCACAGAT

651 GAACTTAACA CATGAATTAA GCCCCAGTTC TGCTGCAGAA AGTCATCGTT

701 CACTCTCAAT TAATGATCCA GATGTGTTGG AGATAACAAA GAATAAGGGA

751 GTAAGGATTC TGAGGCAAGA TTCAGTTCTT GCAGCTTCGA GCAGTGGAGG

801 ATCTGGTGAA CCATGTGCTG TTGCTGCTAT TCGGATGCCA CTGCTTCGTG

851 CTTCGGACTT CAAAGGTGGG ACTCCAGAGT TGGTTGACAC TCGTTATGCC

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 901  ATTTTAGTTT TGGTTCTTTC AAGTGTTGAT GAGAGAGTCT GGAGCTATGA
 951  TGAGATGGAG ATAGTGGAAG TAGTTGCTGA TCAGGTGGCT GTGGCTTTAT
1001  CCCATGCCAC TGTTCTTGAA GAGTCTCAGA CGATGAGGGA GAAACTAGAA
1051  ATGAGAAATC GTGTGCTTCA GCAGGCTCAA GAGAATGCTA TGAAGGCAAG
1101  CCAGGCAAGG ACTTCGTTTC AGAAGGTGAT GAACAATGGT ATGAGGCGGC
1151  CTATGCACTC AATCTTGGGT TTGCTCTCCA TATTTCAAGA TGAGAAAGCC
1201  AGTTCTGACC AGAGGATGAT TGTTGACACA ATGGTGAAAA CAAGCACTGT
1251  TCTCTCAACA CTAATAAATG ACGCAATGGA GATATCTGCA AAAGATGATG
1301  GAAGGTTTCC AGTAGAAATG AAGCCCTTTC AGTTGCATTT ACTGGTCAGG
1351  GAGGCTTCTT GTCTTGTTAA GTGTTTGTGT GTCTATAAGG GATTTGGGTT
1401  TTCTACAGAT GTTCCCACTT CTTTGCCTAA TCAGGTGATG GGCGATGAAA
1451  AGAGAACTTT TCAGGTTTTA TTACATATGG TAGGACACTT ATTAAATGTC
1501  AGCATTGGTA AGGGCTCTGT TATATTCAGA GTCGTTCTAG AGACTGGAGC
1551  TGAGACTGGG AATGACAAAG TTTGGGGAAC AAGAAGACCA AGCACGACAG
1601  ACGAATATGT GACCATAAAA TTTGAAATTG AGGTTAGCCT TGAAGGCTCT
1651  CAATCTGATA GCTCAATCTC GACTATTCAC TTTGGTGGAA GAAGGCATAA
1701  CAGCAAGGAA GTAACGGAGG GCTTGAGTTT CAACATGTGC AAAAAGCTTG
1751  TTCAGGTTGG TTAGAATACT TGATATTGTT TCCCGATTGT TCTTTTTGTT
1801  ATGTTGTCAT TCAGATTGGT TCATCTTGCT AACAAGTATC TGTATATGAT
1851  TAGAGCTCTT GATCATGAAT AAGAAGTAAT GTTCATGGGT TGTGTGATAT
1901  TCATATGTGT TGGCATGTAG GTCTCCCTGT TGGTATTGAC TATGTCTTTA
1951  CCTAGAGAAT TAGGGGTAAG TCTGAGCTGC ATACTATAAT TGTAGTGGTA
2001  GAGAAAAATA GCAAAGCGAG TTCATGGAAG TAGTCTGAAA TGGAGAGAGT
2051  ACACATCATA ATCTAATTTT AGATTGTTAA AATGTGACGA TTTGTAGAAG
2101  TGTACCAAAC ATTTAAACAT CCTAAGGTGG AAAATTCATA AACTTAAAAT
2151  GGTCAGAGGA GTTGCATGTT CTTGCACAAA AATTGTCCCT AGGTTTCTAA
2201  GAAATGCCAC ACAATTTCAA ATTATTTGAG TTGAATTTGA AGGATCTATC
2251  CTACTCCCTT AGGGTTCAGG TAACCGGTAG GATCACCTAT TAAGAGAAAG
2301  AAGGAGAGGA ACTTGAAAAG GGGGTGTTTG AATTGGCTTT TCTAAAAGTA
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2351 TCTTATAAGC TAAAAAGTAA AATCCATAAA TTGGGAATAC TGAATTTTCG

2401 GCTTTTAGCT TATTTTTGTA CTTTTTCAGC CTAAAAGAAA GTGCTTAAAG

2451 GCACTGAGTG TCTTTCCCAA ACACCTTCCA AAAATTAGAA AGAGCTTTAA

2501 AGCCAAAAAC TACTTAAAAT GAGCCAATCC AAACACCCAC TAATTGAAAA

2551 TGTAACCTCC CCTCTTTCTT ATTGACTACT GCTTAGCTGA ATGATCTCAG

2601 AGAATAAGAT GCAGCTTGAG GATTTCTTTC TTGTGCAGAA GATGGTTCTT

2651 TAATACCTCT TCATCTGTAA ATCTTTGGTA AAGAGCTTTG TAGGATGCAA

2701 TATGCCAGGA TGATTTTTGT CCGGTTTCCT CAAGTTTACC TATTAACTGC

2751 CAAATCTTCC ATATGTTCAT ATTATGCTGG CATCAACCTC TTCGTGTTTA

2801 TGTTCTAGTA GAACCAATTC GGAGGGAGAA AGTATATGCA ATTAGAACCC

2851 TCATGACTTG AGAAACTGAA GTTATATTTC TTTAGAACTC TATCTTACCT

2901 ATAAATAAAA TTGAAATAGG GAGAGACCTG AAACCCATGA AGAGACTGAC

2951 TACCAAAAAC ATAGCATGTA AGTCAGCCTT ATACTGACAA GAGTAGGACT

3001 GATTTTTTTA ATTCCTTTTG CTAACAGTGG TGTCCGAGCC ATCTTGGTGC

3051 GCACCCCGAG TCCTCAACTA ATTCATGTGA GACAGGCTAC CTTTCACCAA

3101 CATAGATACC GGGTAACCTT GTTCATAAAA ACTTGGACAG ATGGAATCAT

3151 GGAAAGAAAT CACCTAGGTT TTATTGCCTT CATTGGGCTT TGAACCCGAG

3201 ACCTCATGGT TCTCAACCCA CTTCATTAAC CACTAGGCCA TACATTTGGG

3251 TCCAAAATAT GTACATTTAA TGGGTAAGAT GTTAGTTAGC TTCACCTTAG

3301 TTCCTGATGT GAAACTACAC ACAAGCATCT TCATTCAAAT AATCATTAGA

3351 TAGATATGTA GCATTCACTT CTGCTTGCAC CAGATGTGTG TGCGAGAGGG

3401 TGGGAAAGGA AGGGCTTGGA ATTGGACATA GATGAGAGTA AAAGATGTGT

3451 TCACAAGGAT GCACAGATAA AGATGTTGGC TTCAGTTTAT TTGAGCTGTA

3501 GGAACCAGTC TTTGCTGGTT TGTGGTGTGA CCTCATAGGC TCGTAGCTCT

3551 TAAACTAAAT CCGAAGTGAG CAGGAGAAGT TCAGGATTTC TTCTTTTGAT

3601 ACAAGTTTCT TGGGATTTCT TCTTTTGTAA ATAATATTGG CAGAACTTAT

3651 AAGAAACTCC AATATACACA GAAAATGGTA GATAGACTGG AAACCGGTAC

3701 TTGTATGTAT ATGTTAAATC AGTTAAATAG GAAGGTTGAA GAAGAAAATT

3751 TGCTGGTTGA GCTTCTGAGG CTCAATTTCT CGATATCTAG GTTTAATGTG

3801 AGAAAATAGT AGTTTCTGAC AATTAACTGG TGTATCATAT AAATATAAAG

3851 TAGCTTCTGA CAGTGAGCTG GTGCCTATGT ATCATGTGTT TCAGATATAT

3901 ACTTTCACTG TTTATGTATC ATATAACTTC CCATGCAAAT TGGTACTCCT

3951 TGGGCTTCTG CGGACAGCAT AGCTACATCT TCTCCTTAAT CTTCTTATTA

4001 TTAATATTGG AACTTTAGTG TTGGGATTGA AGTTGCATTT GCTTTCACTG

4051 AATGAACGTT CATAAGAAGT GTGTAATATA AAGGAAGTGT TTTCAGTATG

4101 ATTTCATGCA CTCTGTCTAG GCGAGACCTT GTAAACGAAT CTAATCAGTC

4151 AATTGTCAAG AAGATTTTTA TTCCCGTGTG ACTACAGATC ATGCGATTTT

4201 GTAATTACAA TATCCATCAT TTTCTCCTTT GGGAAGAGGT TTCATCCGTA

4251 GAATTGATTT CTTAATCTGT AAAGTTTTTA GGTGCTTCTT GTAATAACAA

4301 CAATGTACCC AGTGTGATCT CATAAGTGGG GTCTGGGGAG GGTGAGGTGT

4351 ACACATACCT TACCGTTACC TTTGTGGGGT AGAGAGGTTG TTTCCAACAG

4401 ACCCTCGGCT TAGGAAAAAC ATTTTTCAAA ACAACTTTAC GATAGTTGCT

4451 GCTTATGATA TTAATAAATA ATCCAAACAG GAAAACTATT TTGTTCTTGA

4501 GTTTCTTATA TCTGTTATTT GAAGTGATAA GTTGCTTGTG AAGGTGATGA

4551 AGCAACAGCC TGGCTACCAT ATTGCTTATT CTGACCTGTA TTTTTTTTTT

4601 TTTGCATAAA ATTTCAGATG ATGCAGGGAA ATATATGGAT GTCCTCGAAT

4651 GCCCAGGGTC ATGCGCAGGG GATGACACTC ATTCTCAGAT TTCAAAAGCA

4701 GTCATCTTTT AGGAAACGCA TGTTTGAATA CAGAAATCCT TTGGAGCAAC

4751 CGATTTCTAG CACAATGTTC AGAGGCCTTC ATGTACTCCT TACTGATGAC

4801 GATGATGTAA ATAGACTGGT AACTAGAAAG CTCCTCGAAA AACTAGGTTG

4851 CCAAGTAACT GCTGTTTCAA CCGGTTTTCA ATGCCTGAGT GCTCTGGGCC

4901 CTTCACTAAC AACCTTTCAA GTACTCATCT TGGATCTTCA AATGCCAGAA

4951 ATGGATGGAT ATGAAGTGGC ATTGAGGGTG CGTAAGTTCC GCAGCCGTAG

5001 TTGGCCGTTG ATCATAGCCC TGACTGCTAG TTCAGAGGAA CAGGTTTGGG

5051 AGAAATGCCT ACAAGTGGGA ATGAATGGTC TAATAAGGAA ACCTGTTCTT

5101 CTACAAGGCT TGGCTGATGA ACTTCAACGA CTCCTTCAAA GGGGCGGCGG

5151 TGGTGATGGC TTGTGA

LeETR6

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   1  ATGATGAAGA AAGTAGTATC ATGGTTGTTG TTTTTATCGA TCGTTGCTTC
  51  CTTATGGGTT GTTGATGGTT ATATTGAATG TCCTTGTGAT GATAGTGATG
 101  CATTTTTTAG CATGGAAACA ATGTTATTTG TTCAAAAAGC CGGTGATCTC
 151  GGTATAGCAG TGGCCTATTT TTCCATCCCA ATCGAGATCA TCTACTTCGT
 201  TTCCTGTTCT AGTTTTCCGT TCAAATGGGT GCTCTTTCAA TTCGGAGCAT
 251  TCATTGTACT TTGTGGTTTA ACACATTTTC TCACCTTCTT GACTCATTTT
 301  GGCAAATACA CATTTCACCT TATTCTTGCC CTTATTGTTT GCAAACTACT
 351  CACTGCATTA GTCTCGATGC TCACCGCTAT AACACTTATG AATCTCATCC
 401  CTTTGCTGCT TAAAGCCAAG GCAAGGGAGT TTATGCTGAG ACGAAAGAAT
 451  CGTGAGCTTG ATCGAGAAGT TGAAAAAATA AAACAACTAG AGGAACTTGG
 501  ACTGCATGTT AGGATGCTTA CCAATGAGAT CCGCAAGTCA ATTGATCGTC
 551  ATACAATACT CTACACAACT CTTGTTGGGC TGTCGAAGTT ATTGAGTTTG
 601  CAGAATTGTG TTATATGGAT GCCTAATGAG AACAGAACCG AGATGAAACT
 651  GACTCATGAT ACTACAAGGG AGAATGTTTC CAGTGTGTAT AATGTGCCTA
 701  TCCCGATCAG TGATCGAGAA GTAAAAGAGA TCAAGGGGAG TGATGATGTA
 751  AAGATACTTG GTGCAGACTC CCGACTTGCT GCTGCAAGCA GTAGAGGGAG
 801  TTGTGAGCCA GAATCTGTGG CTGCTATTAG GATTCCAATG CTGACGGTCT
 851  CGAATTTCAG AGGTGAAACT CGTGAGATTG TCTCGCAATG TTATGCTATC
 901  CTTGTTTTGG TTCAACCTTG TGGACATGGT AGGTTTTGGC TTAACCAGGA
 951  AGTTGAGATA GTCAGGGCTG CAGCCGATCA AGTTGCTGTG GCGTTGTCCC
1001  ATGCTGCAGT GGTTGAAGAA TCCGAGTATA TCAAAGACAG GTTGATGGAA
1051  CAGAATCAAG CACTGCAGAA AGCAAGAGAG GAAGCTCTTA GAGCAAGTCA
1101  AGCTAGGAGT TCATTTCAGA CGGTTATGAG CCATCGATTG AGAAGACCAA
1151  TGCACTCAAT TTTGGGTCTG CTCTCGATGT TGCAGGAACA GAAGTTACGA
1201  GATGAACAAC AGCTTCTTGT GCATTCTATA ATCAAATCCA GCAATGTTGT
1251  CTCCACCCTG ATGGATGATG TGATAGTTAC TTCAACCAAG GAGAACGTAA
1301  AATTCCCATT GGAAATGAAG CATTTTCAGC TGCATTCCTT GATACGAGAA
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1351 GCTGCTTGTA CCGCCAAATC TTTGTGTATG TATAAGGGTT ACAACATCAC

1401 CATTGAGGTC GAAAAATCAT TTCCTAATAA AGTCATGGGA GACGAAAGAA

1451 GATTTTTTCA AGTTTTGCTT CATATAATTG GAAATCTTTT GAATGGCATC

1501 CACGGAGGAC ATCTTACCTT CAAAGTTCTC TCAGCAAGTG AAAACGATGT

1551 TAGTTGGAAA ACACCGAGAT CAAACTCATC CAATGACATT GTCTATATCA

1601 AGTTCGAGAT TTGCACAAAA TTTAATCGAT CTCAGTCAGA GATCACCCCT

1651 GCTCCTCCAA CATACGACAC TGAGGAGATT GAGGAGAGTT TAAGCTTTGC

1701 TGTTTGCAGG AAGTTGGTTC ATGTAAGCTT TTTATTTTCG TGTTTTTTAG

1751 TACTTCTTGA ATTTTATATC CTTATCTTAG TTAGCTATAT CAATTTTATG

1801 TCCTTATCTG GTGTGTGAAA AATGTTGAAA CAGTTGATGC AAGGAGACAT

1851 CTTTATAATC CGAAATTTAG CAGATTTTGA TCAAGGCATG GCTGTGATTG

1901 TCGGATTCCA AAGGCAGCCG TTAATTCCCT TAGGCATGTC CGAATATGTG

1951 GAGTCTTCTA ATCCCACATA TCCACATCCT GTTTTACGTG GTGTGGAGGT

2001 TCTGTTAGCT GACTATGATG ATTCGAATAG AGCTGTAACA AAGAAGATGC

2051 TCGAGAAATT GGGATGCATC GTTACTTTAG TTTCATCTGG ATATGAATGC

2101 CTCGGTGCTG TTGGCCCCGT TGTGTCCTCG TTACAAATTA TACTTTTGGA

2151 TCTTCATCTG CCTGATTTAG ATGGCTTTGA AGTTACCATG AGACTTCGAA

2201 AGCATAGAAG ACAGACCTGG CCTTTGATCA TCGGTTTAGC TGCAATTACT

2251 GATGAAGATA TCAGAAAATG CCTCAAGATC GGAATGAATG GTATCATCTG

2301 TAAACCATTG CTCTTATCAG GACTCGCCGA TGAGCTTCAG AAGGTTCTGC

2351 TTCATGCAAA CAGAGGAATG CCATGA

APPENDIX B
NEVERRIPE PROMOTER SEQUENCE

The 4768 nucleotides upstream of *NR*'s ATG are shown below. The site of transcription for has been predicted from cDNA clones to be around nucleotide 2015. PLACE Signal Scan (http://www.dna.affrc.go.jp/htdocs/PLACE/signalscan.html) was used to identify *cis*-acting elements in the promoter and leader sequences (Prestridge, 1991; Higo et al., 1999). Elements mentioned in Table 3-1 are shown below, identified by site number ((+) = Current Strand; (-) = Opposite Strand).

Two programs were used to predict the *NR* promoter site:

1. PROSCAN version 1.7 (http://bimas.cit.nih.gov/molbio/proscan/) predicted a promoter region on the forward strand from nucleotide 456 to 706 (underlined region) and a TATA box was found at 670. Promoter Score was 66.75 (cutoff = 53.00) (Prestridge, 1995).

2. TSSP, a promoter prediction software for plant sequences (RegSite Plant DB, Softberry Inc., http://www.softberry.com), predicted three promoters in the given sequence (bold regions) with TATA boxes at nucleotides 303, 1341 and 1769. The site of transcription initiation for these promoters was predicted at nucleotide 326, 1375, and 1783, respectively.

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1       CTAAAAGGGGGATTAGTTCTTATTTTTAATTAAATTACTGTATCCAAAAG
         (-)S000259
                                                  (+)S000403
                                                  (-)S000180

51      TATAAGTAAATAGGTTTAATATCAAAGCAAATTTAATTAATCTTGTTAGG
                                                    (-)S000439

101     GTACTATGTCATGTTCCGTCACTTCATGTTCAAATAACAAATAAAAAAGA
                     (-)S000024
                                          (+)S000439
                                          (+)S000181

151     AGAATCTAGACTCGTGGGGAGGTTAGTGGAAGACACTAGAATTCTCTAAG
                                        (+)S000292
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201    TCTTTCCTCGGAAGACTCTAGAATATTTTAGATATTTCCTAGAAAAGTTT
                     (-)S000392
                                    (-)S000198
                                           (+)S000245

251    TGGAAAATCCTAGAACGTGTAGAGAATTCTAGAGAGGCATCAAAGTGTAA
       (+)S000198
        (+)S000198
                 (+)S000414
                 (+)S000415
                 (-)S000415

301    AT**ATTTTAGGGACTTGTGTAGTAACT**TTTATTTACACTTAGACTCCTAAA
               (-)S000292
                                                    (-)S000259

351    AGGTAATATAAACAGAGACACTCTCATTCTAAATCACCGAATAAAGTTGT
                 (-)S000270
                                              (-)S000273

401    AAGCAATATTTGAAGCTTAATACAAAACATTCTTTCAAACTTCCAAATCT
                                                      (-)S000245

451    TTCTAAAAAGTCTAGATTCAAAGAACTTACTTAAGTTTAGCACAAAATAT

501    TAGTAAGACTCTTTGTGATACTTAAAGCCATCAAATTTAATTGAAGGACT

551    TGTCAATTAAACTTGACATCCCTAGGATTAAACAAAATCAATAATATAAT
       (-)S000390
                (+)S000390
                                                    (-)S000030

601    TGGCTCATATGGTTGTTGAATTGGCATTATTGTTGAACCACAAATTAACA
           (+)S000370
           (+)S000407
           (-)S000370
           (-)S000407
                   (-)S000314
                         (-)S000030
                                     (-)S000314
                                                      (-)S000256

651    TGGATAATGATATTTTGAATTATATATGGATAAATATTCTATGACTAAGG
      (-)S000403
       (+)S000180
        (+)S000198
        (+)S000199
                               (-)S000256
                                (-)S000403
                                 (+)S000180
                                  (+)S000198
                                  (+)S000199
                                        (+)S000392
                                               (+)S000442
                                                      (+)S000198
                                                      (-)S000298
                                                       (+)S000198
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701    AAAAAATGACAATTAAAATTAATTTGATTAGATGTTTATTTTTCTAGAAT
                                              (-)S000198
                                                (-)S000245

751    TTTATGATAATATTTTGCGATTGTTATAATATTTATCTGATCTTTCGAAT
           (+)S000198
           (+)S000199
                                     (-)S000198
                                      (-)S000199

801    ATTTTTACATAATGGTTAGTATGTATATTTTTAATCATATTTCTTTACCT
                  (-)S000408
                  (-)S000175
                                                (-)S000245
                                                    (-)S000198

851    TTCGAGTAAACACTATGTGATAAAAACTTTAAGAAATAAATATCAATTAT
                        (+)S000198
                        (+)S000199
                               (+)S000273
                                     (+)S000245

901    AAATTACGATTATATGTTATCGATCATGATATTCATGATTATTAGCTTTT
                       (-)S000199

951    GCTGAGTCCATAGGTTTGTGTTCTATTTATCAATGTTTACATAAAAGATA
                                (-)S000198
                                 (-)S000199
                                                    (+)S000198
                                                    (+)S000199

1001   ATGTTTAATCCTTAATTCGTATATAATTCTACATTCATTTAATTTCAAAA

1051   TGGTAACCCTATAAGTTTCTACTAAAATTCCGATAAAAATAGTAAGTTTA
                      (-)S000245
                                     (+)S000198
                                     (+)S000199

1101   ATCAATCAATTAATATTCCTTTTTCAAATTAAAACATTAATCAATAGAGT
                       (+)S000259
                         (-)S000198
                                                    (-)S000421

1151   TGTAAGTAACCATACCTCTTAAAACTTTGGGCTTGGAATGAGAAAATGAG
            (+)S000408
                                                  (+)S000245
                                                   (+)S000198

1201   GTAAGCTTAGAAAATGTATTTGGTGTTTTAAAGTTGTAATTTTTCTAATC
              (+)S000245
               (+)S000198
                                  (-)S000273
                                                  (-)S000198
                                                    (-)S000245

1251   AATAAAATAGATATCAAAAGTCTTTAAATTAATTGGATGAAAATGCTACA
                                               (+)S000198
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1301   TGTTCGAAATTTAAAGAAAATCGTGAATATAATTAGATAA**AATAATATTA**
                  (+)S000245
                   (+)S000198
                                          (+)S000198
                                          (+)S000199

1351   **ATGATGAAATTGAAGACAATATAAT**ACAATTGAAATCAATTCCATATAAA
        (-)S000252
                               (+)S000407
                               (-)S000407

1401   AAGATACTTATCAAACTAGTCTTTAAATTAATTGGATGAAAACGCTACAT
            (-)S000124
             (-)S000199

1451   GTTCGGAATTTAAAGAAAATTGTGAATATAATTAGAGAAAACAATACTAA
                (+)S000245
                 (+)S000198
                                          (+)S000245
                                                 (+)S000185

1501   TGATAAAACTGAAGACAATATAATACAGTTGAAATCAATTACATATAAGA
       (+)S000198
       (+)S000199
                              (+)S000176
                              (+)S000407
                              (-)S000409
                              (-)S000407
                                                        (+)S000245

1551   AAACACTTACTAAACTTTTCATGTTTTTTCGTTGTTATTTCAAAAGACAA
                             (-)S000198
                                                        (+)S000148
                                                        (+)S000263

1601   ACACCAAACTTAAAAGTAACAAGAAAAATGAGAGATGAATTTTAACTAGA
       (+)S000167
                     (+)S000439
                          (+)S000245
                           (+)S000198

1651   TTTAAGTTTTCATAAAAAAATAAAATACTACAATTGTATTTTATTAAATT
                                    (+)S000407
                                    (-)S000407

1701   GTCTAAAATATTTTTATCGTGTTAAAAGAAAAAGTCATTTTACCTCAGAG
                  (-)S000198
                   (-)S000199
                                (+)S000245
                                 (+)S000198
                                            (-)S000198
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1751   AATTTATTAATCCAATAA**TATTAATTAACTGAG**CTACTAATATTCTCGGT
                (+)S000030
                                (+)S000177
                                (+)S000409
                                (-)S000176
                                                    (+)S000392
                                                       (-)S000401

1801   GATCATAAATATGACCACCAACTAAAAAAAAAATGTTAACACATGTATGAA
                                                 (+)S000292
                                                  (+)S000174
                                                  (+)S000407
                                                  (-)S000144
                                                  (-)S000413
                                                  (-)S000407

1851   GCTCTTCACTGTAGCAGCGTGATTCCGGTGAGTAGTTTAAGGCTTTTTTT
                                                              (+)S000298
                                                               (-)S000198
                                                                 (-)S000198

1901   TTTCCTCTTAATTACTTCCCTCCACTTTTAGTTGGGGAGCTTTTCTCGAT
                                                       (-)S000245
                                                             (+)S000198
                                                             (+)S000199

1951   AATCGCCAAATTTCCATAAATTCAAATCAGTATATCATCGAAGAACACGA
                (-)S000198

2001   CGAAAATCCGATGGCCACAAGCAAAACGACAGTTCAAATTCACGGAGATT
       (+)S000198

2051   GTGAAAATGATAAAGTGAAGTTACGTGGAGTAGTAGTTCAGTGAAGTAGT
         (+)S000198
               (+)S000198
               (+)S000199
                 (-)S000273
                             (+)S000414
                             (+)S000415
                             (-)S000415

2101   AGATACTGAGATCGCATTCTCCGTCGTCATTTTTCACATCGAAATAGTAA
                                    (-)S000198

2151   TCTCTTTCTCAGATTTGTACTTTTTTTTTGAGTTTCTCGTTGTTTTCTCC
          (-)S000245
                                         (-)S000245
                                                    (-)S000245

2201   GCTTGAATTGATACTGATCTGATTTTCATGTTTTAGGTCGTGTAAAAAAA
                            (-)S000198

2251   TGAAAAAATTGCTGCGAGACAGGTATGTGTCGCAGCAGGAAATAGCATCT
       (+)S000198
                     (-)S000270
                                              (+)S000198
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2301   TAAAGGAAGGAAGGAAGGAAACTCGAAAGTTACTAAAAATTTTTGGTAAT
                                                  (+)S000198

2351   TAGTTGCTATGCCTGAAGTATTTAAGTTTCGGTTTGGCTGTAGATTTTTG
                              (-)S000250

2401   AAGTTGAAACGTAAAAATTTGAGTTTTTGGAGTTGTGATTTATGGAATTT
              (+)S000415
              (-)S000415
                                 (-)S000421

2451   GAAGTTGTGTTTAGGTATAGATTTTATTTGTAAAAAAATTGAAGTTTTGT

2501   GAGTATAAGTACCCCCAAAAATTGAAAATATTTGAAGATTAGATTTTCAA
                           (+)S000198
                                                 (-)S000198

2551   AATTTGATCAAATACATATATGAAGATAGATTTTAAAAATCTGTGGCAAA

2601   ATGCTAGCTAAATTATCTGTTATTTATGTTTCTCTATGGTGTAACTTCTT
                 (-)S000198
                  (-)S000199
                      (+)S000176
                                    (-)S000245
                                                        (-)S000198

2651   TTTCTTCTTTTGTTAAATGGAATTTGATTTTGAACTGTAAATTGTAGCTT
      (-)S000245
              (-)S000439
              (-)S000181

2701   TATTTTCGATGTCCAAGGGTTTTTCAATTTGCAGATAATAGAAAGAGTGA
       (-)S000198
                         (-)S000198
                                          (+)S000198
                                          (+)S000199
                                                (+)S000245

2751   ATTTGATTCTACCATGGTTTTGCTATTAGTGCATAAATTCCTTCCAAGTT
                    (-)S000408

2801   GCTATTTTGATTGGGGAGAAGGATATGTGTGCTTATCTATTACTTAGAGG
               (-)S000030
                           (+)S000180
                                     (-)S000124
                                      (-)S000199
                                                        (+)S000198

2851   AAATGAAATTGAGTGTGAATTTGAATTGGCATGGATCCATAACCTTTTAG
                               (-)S000030
                                                 (+)S000259

2901   ATGCATAATCTTGTAGCCTCATGGAGGTTGAAAACCCAGAGTTGCCATCT
                                               (-)S000421
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2951   AATAGATTAATCATCAGAAAACAAGCAGGTATACCTTTTGGTAAGTTCAG
                  (+)S000245
                                        (+)S000259

3001   GAGATAAAAAGGTATTTTCTTGCCTTGTTTTTCTTTTCGGCGTGGTAGTT
        (+)S000198
        (+)S000199
            (-)S000259
                   (-)S000198
                     (-)S000245
                                   (-)S000198
                                     (-)S000245
                                          (-)S000250

3051   GAAATGTTTAACGATGGAAATGAATGTAAATTGTACCATAATTACGTAGG
                  (+)S000198
                                                  (+)S000415
                                                  (-)S000415

3101   AGCATTCCCTCTGGAGAAATCCTATAATAGGGCAAACACCAAAAATAGCT
                (+)S000245
                                       (+)S000148
                                       (+)S000263

3151   TGGAGTTGGAGAAAAACCATGCTCTACTTGGTTTCTTGTTATTCATTCTT
        (-)S000421
               (+)S000245
                (+)S000198
                   (+)S000408
                                  (-)S000408
                                     (-)S000245
                                        (-)S000439

3201   TCCGTATTACTATGAAAGCTTAGATATTGTAGACAGTGAATTCAAGTAAA

3251   CTATTCCTTCCGTCGGAAGATCAAGAAATTGCTACTCCCTCCGTTTCATT
                 (-)S000153
                             (+)S000245

3301   ATACTTGGCACCTATTCTAGAAATGAATTTTTCTTTTTACTAGTCCATTT
                  (+)S000392
                        (+)S000245
                                  (-)S000198
                                    (-)S000245

3351   TAGCAAACCAAGAGAATTTTATTGTTTTCTTTCTATACTACCCTTAGAAC
          (+)S000408
                                (-)S000245
                                    (-)S000245

3401   TCTCTCTAGTCCATATTGAGTGAAATTTTAGGGGTAATTGTTCAAAGTTC
                                       (+)S000198
                                                        (+)S000314

3451   AACATAATTTTTTAAACTTCTTTCCATATTTGCACTTCTCTTTAATGAGG
```

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3501   TTCTTAGCTATACTTTATATTTTCTAGGATATAAATTGCACAACTTACAA
                 (+)S000273
                        (-)S000198
                          (-)S000245
                                 (+)S000180

3551   AGTTGTATTTACGATTTCACTTTGAATCATAAATATAACTTTGTAAGTAG

3601   TGCAATTTATCTCCTAGAAAATGTAAATATAGCTAAGAACCTCATTAAAG
           (-)S000198
            (-)S000199
                     (+)S000245
                      (+)S000198

3651   GAAAGGACAAATATAGAAAAAAGTTTCAACAATTATCTTGAACTTTGTAT
                    (+)S000245
                     (+)S000198
                                (+)S000314
                                     (-)S000198
                                      (-)S000199

3701   AGTTTCACTTATACTGAACTATGAAGAAAATCCCAAAAATTTCACTTAAT
                               (+)S000245
                                (+)S000198

3751   ATGGACTAGAGAGAATACCACTTTAAATTTTTCATTTTTTCAATCTATAT
                 (-)S000392
                        (+)S000273
                                (-)S000198
                                        (-)S000198

3801   TTGAAATCGAAAAGTGAATTAAGAGAGCTAAGATTTCATGTATCTTGATG
                                                      (-)S000314

3851   TTGAAGACACTGCTAAAATAGTTCACTACTTTGATGTTGAAGACTCTGTT
                                           (-)S000314
                                                     (+)S000176
                                                      (-)S000314

3901   GATGCTGTAGAAATTTTCTGGTTGAATTTTGAGGTTTATTATTGTGCTGA
              (+)S000245
                   (-)S000198
                     (-)S000245

3951   AATTTTCAATTTAAATGTTTGGCTCCAATTGTACAAACTTTTCATCATGA
       (-)S000198
                               (+)S000030
                                (+)S000407
                                (-)S000407

4001   AACTAATGTCAATCGCTTCAATACAAAACCAAAAGTTTGATGTCATGCAG
             (-)S000390
                               (+)S000408
                                                   (+)S000264
```

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4051   TGTAAAATTTTTACGTCGGGATTTCAAATTGCTTCCTTAACTTATACGAG
                 (+)S000415
                 (-)S000415
                   (-)S000153

4101   TAATTATCATAATCAAATCACTAAACCTTTTAGGTAATGACTCTTTCTAC
        (-)S000198
         (-)S000199
                              (+)S000259
                                     (+)S000198
                                                 (-)S000245

4151   TTTTAAGAATAGTTTGGGAATAAGCAAAAGGGCAATAATGGAAAGTAGAC
           (-)S000392
                              (-)S000259

4201   TTTGAACTATGTTCTTGAATGTGTTTCCTAAGGAGTGTGCCAAATCCAAA

4251   AATTTCGCTCAATATGAATTAGAGGAAGCATTAAATAACCACAAATTGTA
                                        (+)S000408

4301   GTAGTAGTCAAATTTAAAGTTTCAACTGTCATCAATAAGTTTAGTTTTAA
           (-)S000310
            (-)S000390
                         (+)S000409
                         (+)S000407
                         (-)S000176
                         (-)S000407

4351   ATACACCCCTGTAAAAGCTTTCTTAAGGGTTGTGCCTAATCAACATGGGT
                       (-)S000245
                                            (+)S000314
                                                   (-)S000142
                                                    (-)S000390

4401   CAAGTAAAATGAAACAAAGGAAATATTATTTTAGAATAACTGTGACAGCG
                       (+)S000198
                                        (-)S000392
                                            (+)S000177
                                            (+)S000409
                                            (-)S000176
                                                 (-)S000422

4451   TAGGAAGCTTCTAATTTCTTTCCTTAAACTGCTAACTAGTGGTAATATTA
                    (-)S000245
                                                 (+)S000198

4501   AAGACACAGATCAGTTTTTTTGGTTGCAATGATCAGGAACACTTGTATAA
                                               (+)S000292
                                                (+)S000407
                                                (-)S000407
```

```
4551   ACGTAAAAGCAATAACTAACAAATGTATGTCCACTAACGTTTATTAATGT
     (+)S000415
     (-)S000415
                   (+)S000439
                   (+)S000181
                      (+)S000407
                      (-)S000407
                                        (+)S000415
                                        (-)S000415
                                                   (+)S000270

4601   CTCTTCATTCATTATTTTTTCCCTCGAACTGCGATAAACAAATGTGTGCA
                 (+)S000298
                  (-)S000198
                   (-)S000198
                                    (+)S000198
                                    (+)S000199
                                          (+)S000407
                                          (-)S000407

4651   CTGCACGACAAAGATGTCGTGGACAATGGTGTATGCACTTTTTATTGTCA

4701   GTTTTACTATTTATGAAGAACCATTCCTCATTCTGTCTTTTTCAGATTCT
                                         (-)S000198

4751   TTGGGACGAAACGAGATA
```

APPENDIX C
SEQUENCE ALIGNMENT OF THE KINASE DOMAINS OF THE ARABIDOPSIS ETHYLENE RECEPTORS.

All five Arabidopsis ethylene receptor sequences were aligned using Pretty and the consensus sequence for 4 out of 5 sequences was determined. The conserved motifs for histidine kinases as described by Parkinson and Kofoid (1992) are noted, where: ◊, non polar (I,L,M,Q); ♦, polar (A,G,P,S,T); +, basic (H,K,R); -, acidic/amidic (D,E,N,Q). The amino acids chosen for mutagenesis are in bold.

```
                                H box
ERS1_kinase EAEMAVHARN DFLAVMNHEM RTPMHAIISL SSLLLETE.L SPEQRVMIET
ETR1_kinase EAETAIRARN DFLAVMNHEM RTPMHAIIAL SSLLQETE.L TPEQRLMVET
EIN4_kinase NAMMASQARN TCQKVMSHGM RRPMHTILGL LSMF.QSESM SLDQKIIVDA
ETR2_kinase DALRASQARN AFQKTMSEGM RRPMHSILGL LSMI.QDEKL SDEQKMIVDT
ERS2_kinase NALRANQAKA AFEQMMSDAM RCPVRSILGL LPLILQDGKL PENQTVIVDA
  Consensus -A--A--ARN -F---M---M R-PMH-I--L -S-----E-L ---Q---V--
                           ◊♦HE◊ +-PL

ERS1_kinase ILKSSNLVAT LISDVLDLSR LEDGSLL.LE NEPFSLQAIF EEVISLIKPI
ETR1_kinase ILKSSNLLAT LMNDVLDLSR LEDGSLQ.LE LGTFNLHTLF REVLNLIKPI
EIN4_kinase LMKTSTVLSA LINDVIDISP KDNGKS.ALE VKRFQLHSLI REAACVAKCL
ETR2_kinase MVKTGNVMSN LVGDSMDV.. .PDGRF.GTE MKPFSLHRTI HEAACMARCL
ERS2_kinase MRRTSELLVQ LVNNAGDIN. ..NGTIRAAE THYFSLHSVV KESACVARCL
  Consensus --K-S----- L--D--D--- ---G-----E ---F-LH--- -E--------

                                        N box
ERS1_kinase ASVKKLSTNL ILSADLPTYA IGDEKRLMQT ILNIMGNAVK FT.KEGYISI
ETR1_kinase AVVKKLPITL NLAPDLPEFV VGDEKRLMQI ILNIVGNAVK FS.KQGSISV
EIN4_kinase SVYKGYGFEM DVQTRLPNLV VGDEKRTFQL VMYMLGYILD ..MTDGGKTV
ETR2_kinase CLCNGIRFLV DAEKSLPDNV VGDERRVFQV ILHMVGSLVK PRKRQEGSSL
ERS2_kinase CMANGFGFSA EVYRALPDYV VGDDRKVFQA ILHMLGVLMN RKIK...GNV
  Consensus ---------- -----LP--V VGDE-R--Q- IL---G---- ----------
                                    ◊.Q◊ ◊◊N◊◊.NA
                                                      G1 box
ERS1_kinase IASIMK.... ..PESLQELP SPEFFPVLSD SHFYLCVQVK DTGCGIHTQD
ETR1_kinase TALVTK.... ..SDT....R AADFFVVPTG SHFYLRVKVK DSGAGINPQD
EIN4_kinase TFRVICE.GT GTSQDKSKRE TGMWKSHMS. .DDSLGVKFE VEINEIQNPP
ETR2_kinase MFKVLKE..R G.SLDRSDHR WAAWRSPASS ADGDVYIRFE MNVENDDSSS
ERS2_kinase TFWVFPESGN SDVSERKDIQ EAVWRHCYSK EYMEVRFGFE VTAEGEESSS
  Consensus ---V------ ---------- --------S- ---------- ----------
                                                    ◊. D-G♦G◊♦
```

```
                F box              G2 box
ERS1_kinase IPLLFTKFVQ PRTGTQRNHS GGGLG....L ALCKRFVGLM GGYMWI..ES
ETR1_kinase IPKIFTKFAQ TQSLATRSSG GSGLG....L AISKRFVNLM EGNIWI..ES
EIN4_kinase LDGSAMAMR. .HIPNRRYHS N.GIKEGLSL GMCRKLAQMM QGNIWISPKS
ETR2_kinase QSFASVSSRD QEVGDVRFSG GYGLGQDLSF GVCKKVVQLI HGNISVVPGS
ERS2_kinase SSSGSNLEEE EENP...... ........SL NACQNIVKYM QGNIRVVEDG
  Consensus ---------- ------R--- --G------L --C---V--M -GNI-----S
                ◊F-PF              G♦GLG    L

ERS1_kinase EGLEKGCTAS FIIRLGICNG PSSSSGSMAL HLAAKSQTRP WNW~
ETR1_kinase DGLGKGCTAI FDVKLGISER SNESKQSGIP KVPAIPRHSN FTG~
EIN4_kinase HGQTQSMQLV LRFQTRPSIR .RSILAGNAP ELQ.HPNSNS ILRG
ETR2_kinase DGSPETMSLL LRFRRRPSIS VHGSSESPAP DHHAHPHSNS LLRG
ERS2_kinase LGLVKSVSVV FRFQLRRSMM SRGGGYSGET FRTSTPPSTS H~~~
  Consensus -G-------- -------S-- ------S--- -----P---- ----
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LIST OF REFERENCES

Abel, S., and Theologis, A. (1994). Transient transformation of Arabidopsis leaf protoplasts--a Versatile Experimental System to Study Gene-Expression. Plant J. **5,** 421-427.

Abeles, F.B., Morgan, P.W., and Saltveit, M.E. (1992). Ethylene in plant biology. (San Diego: Academic Press).

Ahmad, M., Jarillo, J.A., Smirnova, O., and Cashmore, A.R. (1998). The CRY1 blue light photoreceptor of Arabidopsis interacts with phytochrome A in vitro. Mol. Cell. **1,** 939-948.

Alberts, B. (2002). Molecular biology of the cell. (New York: Garland Science).

Alonso, J.M., and Ecker, J.R. (2001). The ethylene pathway: a paradigm for plant hormone signaling and interaction. Sci. STKE **2001,** RE1.

Alonso, J.M., Hirayama, T., Roman, G., Nourizadeh, S., and Ecker, J.R. (1999). EIN2, a bifunctional transducer of ethylene and stress responses in Arabidopsis. Science **284,** 2148-2152.

Aravind, L., and Ponting, C.P. (1997). The GAF domain: an evolutionary link between diverse phototransducing proteins. Trends Biochem. Sci. **22,** 458-459.

Atta-Aly, M.A., Brecht, J.K., and Huber, D.J. (2000). Ethylene feedback mechanisms in tomato and strawberry fruit tissues in relation to fruit ripening and climacteric patterns. Postharvest Biol. Tech. **20,** 151-162.

Bilwes, A.M., Alex, L.A., Crane, B.R., and Simon, M.I. (1999). Structure of CheA, a signal-transducing histidine kinase. Cell **96,** 131-141.

Bleecker, A.B., Estelle, M.A., Somerville, C., and Kende, H. (1988). Insensitivity to ethylene conferred by a dominant mutation in Arabidopsis thaliana. Science **241,** 1086-1089.

Bleecker, A.B., and Kende, H. (2000). Ethylene: a gaseous signal molecule in plants. Annu. Rev. Cell. Dev. Biol. **16,** 1-18.

Cancel, J.D., and Larsen, P.B. (2002). Loss-of-function mutations in the ethylene receptor ETR1 cause enhanced sensitivity and exaggerated response to ethylene in Arabidopsis. Plant Physiol. **129,** 1557-1567.

Chae, H.S., Faure, F., and Kieber, J.J. (2003). The eto1, eto2, and eto3 mutations and cytokinin treatment increase ethylene biosynthesis in Arabidopsis by increasing the stability of ACS protein. Plant Cell **15,** 545-559.

Chang, C., Kwok, S.F., Bleecker, A.B., and Meyerowitz, E.M. (1993). Arabidopsis ethylene-response gene Etr1--similarity of product to 2-component regulators. Science **262,** 539-544.

Chang, C., and Meyerowitz, E.M. (1995). The ethylene hormone response in Arabidopsis--a eukaryotic 2-component signaling system. Proc. Natl. Acad. Sci. U. S. A. **92,** 4129-4133.

Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W., and Ecker, J.R. (1997). Activation of the ethylene gas response pathway in Arabidopsis by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. Cell **89,** 1133-1144.

Chen, Q.H.G., and Bleecker, A.B. (1995). Analysis of ethylene signal-transduction kinetics associated with seedling-growth response and chitinaseinduction in wild-type and mutant Arabidopsis. Plant Physiol. **108,** 597-607.

Chen, Y.F., Randlett, M.D., Findell, J.L., and Schaller, G.E. (2002). Localization of the ethylene receptor ETR1 to the endoplasmic reticulum of Arabidopsis. J. Biol. Chem. **277,** 19861-19866.

Ciardi, J.A., Tieman, D.M., Jones, J.B., and Klee, H.J. (2001). Reduced expression of the tomato ethylene receptor gene LeETR4 enhances the hypersensitive response to Xanthomonas campestris pv. vesicatoria. Mol. Plant Microbe Interact. **14,** 487-495.

Ciardi, J.A., Tieman, D.M., Lund, S.T., Jones, J.B., Stall, R.E., and Klee, H.J. (2000). Response to Xanthomonas campestris pv. vesicatoria in tomato involves regulation of ethylene receptor gene expression. Plant Physiol. **123,** 81-92.

Clark, K.L., Larsen, P.B., Wang, X.X., and Chang, C. (1998). Association of the Arabidopsis CTR1 Raf-like kinase with the ETR1 and ERS ethylene receptors. Proc. Natl. Acad. Sci. U. S. A. **95,** 9060-9060.

Cosgrove, D.J., Gilroy, S., Kao, T.H., Ma, H., and Schultz, J.C. (2000). Plant Signaling 2000. Cross talk among geneticists, physiologists, and ecologists. Plant Physiol. **124,** 499-506.

D'Agostino, I.B., Deruere, J., and Kieber, J.J. (2000). Characterization of the response of the Arabidopsis response regulator gene family to cytokinin. Plant Physiol. **124,** 1706-1717.

Davie, J.R., Wynn, R.M., Meng, M., Huang, Y.S., Aalund, G., Chuang, D.T., and Lau, K.S. (1995). Expression and characterization of branched-chain alpha-ketoacid dehydrogenase kinase from the rat. Is it a histidine-protein kinase? J. Biol. Chem. **270,** 19861-19867.

Davies, P.J. (1995). Plant hormones: physiology, biochemistry, and molecular biology. (Dordrecht ; London: Kluwer Academic).

Dutta, R., and Inouye, M. (2000). GHKL, an emergent ATPase/kinase superfamily. Trends Biochem. Sci. **25,** 24-28.

Emanuelsson, O., Nielsen, H., Brunak, S., and von Heijne, G. (2000). Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. J. Mol. Biol. **300,** 1005-1016.

Fankhauser, C., Yeh, K.C., Lagarias, J.C., Zhang, H., Elich, T.D., and Chory, J. (1999). PKS1, a substrate phosphorylated by phytochrome that modulates light signaling in Arabidopsis. Science **284,** 1539-1541.

Gamble, R.L., Coonfield, M.L., and Schaller, G.E. (1998). Histidine kinase activity of the ETR1 ethylene receptor from Arabidopsis. Proc. Natl. Acad. Sci. U. S. A. **95,** 7825-7829.

Gamble, R.L., Qu, X., and Schaller, G.E. (2002). Mutational analysis of the ethylene receptor ETR1. Role of the histidine kinase domain in dominant ethylene insensitivity. Plant Physiol. **128,** 1428-1438.

Gao, Z., Chen, Y.F., Randlett, M.D., Zhao, X.C., Findell, J.L., Kieber, J.J., and Schaller, G.E. (2003). Localization of the Raf-like kinase CTR1 to the endoplasmic reticulum of Arabidopsis through participation in ethylene receptor signaling complexes. J. Biol. Chem. **278,** 34725-34732.

Gaspar, T., Kevers, C., Faivre-Rampant, O., Crevecoeur, M., Penel, C., Greppin, H., and Dommes, J. (2003). Changing concepts in plant hormone action. In Vitro Cell. Dev. Biol. Plant **39,** 85-106.

Glazebrook, J. (2001). Genes controlling expression of defense responses in Arabidopsis--2001 status. Curr. Opin. Plant Biol. **4,** 301-308.

Guzman, P., and Ecker, J.R. (1990). Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. Plant Cell **2,** 513-523.

Hall, A.E., and Bleecker, A.B. (2003). Analysis of combinatorial loss-of-function mutants in the Arabidopsis ethylene receptors reveals that the ers1 etr1 double mutant has severe developmental defects that are EIN2 dependent. Plant Cell **15,** 2032-2041.

Hall, A.E., Chen, Q.H.G., Findell, J.L., Schaller, G.E., and Bleecker, A.B. (1999). The relationship between ethylene binding and dominant insensitivity conferred by mutant forms of the ETR1 ethylene receptor. Plant Physiol. **121,** 291-299.

Hall, A.E., Findell, J.L., Schaller, G.E., Sisler, E.C., and Bleecker, A.B. (2000). Ethylene perception by the ERS1 protein in Arabidopsis. Plant Physiol. **123,** 1449-1457.

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

Hirayama, T., Kieber, J.J., Hirayama, N., Kogan, M., Guzman, P., Nourizadeh, S., Alonso, J.M., Dailey, W.P., Dancis, A., and Ecker, J.R. (1999). Responsive-to-antagonist1, a Menkes/Wilson disease-related copper transporter, is required for ethylene signaling in Arabidopsis. Cell **97,** 383-393.

Hua, J., Chang, C., Sun, Q., and Meyerowitz, E.M. (1995). Ethylene insensitivity conferred by Arabidopsis Ers gene. Science **269,** 1712-1714.

Hua, J., and Meyerowitz, E.M. (1998). Ethylene responses are negatively regulated by a receptor gene family in Arabidopsis thaliana. Cell **94,** 261-271.

Hua, J., Sakai, H., Nourizadeh, S., Chen, Q.H.G., Bleecker, A.B., Ecker, J.R., and Meyerowitz, E.M. (1998). EIN4 and ERS2 are members of the putative ethylene receptor gene family in Arabidopsis. Plant Cell **10,** 1321-1332.

Huang, Y., Li, H., Hutchison, C.E., Laskey, J., and Kieber, J.J. (2003). .Biochemical and functional analysis of CTR1, a protein kinase that negatively regulates ethylene signaling in Arabidopsis. Plant J. **33,** 221-233.

Hutchison, C.E., and Kieber, J.J. (2002). Cytokinin signaling in Arabidopsis. Plant Cell **14 Suppl,** S47-59.

Hwang, I., and Sheen, J. (2001). Two-component circuitry in Arabidopsis cytokinin signal transduction. Nature **413,** 383-389.

Inoue, T., Higuchi, M., Hashimoto, Y., Seki, M., Kobayashi, M., Kato, T., Tabata, S., Shinozaki, K., and Kakimoto, T. (2001). Identification of CRE1 as a cytokinin receptor from Arabidopsis. Nature **409,** 1060-1063.

Kende, H., and Zeevaart, J.A.D. (1997). The five "classical" plant hormones. Plant Cell **9,** 1197-1210.

Kieber, J.J., and Ecker, J.R. (1993). Ethylene gas--its not just for ripening any more. Trends Genet. **9,** 356-362.

Kieber, J.J., Rothenberg, M., Roman, G., Feldmann, K.A., and Ecker, J.R. (1993). Ctr1, a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of the Raf family of protein-kinases. Cell **72,** 427-441.

Koretke, K.K., Lupas, A.N., Warren, P.V., Rosenberg, M., and Brown, J.R. (2000). Evolution of two-component signal transduction. Mol. Biol. Evol. **17,** 1956-1970.

Lapko, V.N., Jiang, X.Y., Smith, D.L., and Song, P.S. (1999). Mass spectrometric characterization of oat phytochrome A: isoforms and posttranslational modifications. Protein Sci. **8,** 1032-1044.

Lashbrook, C.C., Tieman, D.M., and Klee, H.J. (1998). Differential regulation of the tomato ETR gene family throughout plant development. Plant J. **15,** 243-252.

Lincoln, J.E., Cordes, S., Read, E., and Fischer, R.L. (1987). Regulation of gene-expression by ethylene during Lycopersicon esculentum (tomato) fruit-development. Proc. Natl. Acad. Sci. U. S. A. **84,** 2793-2797.

Lincoln, J.E., and Fischer, R.L. (1988). Diverse mechanisms for the regulation of ethylene-inducible gene-expression. Molec. Gen. Genet. **212,** 71-75.

Liu, G.Z., Pi, L.Y., Walker, J.C., Ronald, P.C., and Song, W.Y. (2002). Biochemical characterization of the kinase domain of the rice disease resistance receptor-like kinase XA21. J. Biol. Chem. **277,** 20264-20269.

Maeda, T., Wurgler-Murphy, S.M., and Saito, H. (1994). A two-component system that regulates an osmosensing MAP kinase cascade in yeast. Nature **369,** 242-245.

Maeda, T., Takekawa, M., and Saito, H. (1995). Activation of yeast PBS2 MAPKK by MAPKKKs or by binding of an SH3-containing osmosensor. Science **269,** 554-558.

Mattoo, A., and Suttle, J.C. (1991). The plant hormone ethylene. (Boca Raton, FL: CRC Press).

Mccormick, S., Niedermeyer, J., Fry, J., Barnason, A., Horsch, R., and Fraley, R. (1986). Leaf disk transformation of cultivated tomato (Lycopersicon esculentum) using Agrobacterium tumefaciens. Plant Cell Rep. **5,** 81-84.

Mukhopadhyay, M.J., and Sharma, A. (1991). Manganese in cell-metabolism of higher-plants. Bot. Rev. **57,** 117-149.

Nollen, E.A., and Morimoto, R.I. (2002). Chaperoning signaling pathways: molecular chaperones as stress-sensing 'heat shock' proteins. J. Cell Sci. **115,** 2809-2816.

O'Donnell, P.J., Jones, J.B., Antoine, F.R., Ciardi, J., and Klee, H.J. (2001). Ethylene-dependent salicylic acid regulates an expanded cell death response to a plant pathogen. Plant J. **25,** 315-323.

O'Donnell, P.J., Schmelz, E.A., Moussatche, P., Lund, S.T., Jones, J.B., and Klee, H.J. (2003). Susceptible to intolerance--a range of hormonal actions in a susceptible Arabidopsis pathogen response. Plant J. **33,** 245-257.

O'Neill, S.D., Nadeau, J.A., Zhang, X.S., Bui, A.Q., and Halevy, A.H. (1993). Interorgan regulation of ethylene biosynthetic genes by pollination. Plant Cell **5,** 419-432.

O'Sullivan, W.J., and Smithers, G.W. (1979). Stability constants for biologically important metal-ligand complexes. Methods Enzymol. **63,** 294-336.

Ota, I.M., and Varshavsky, A. (1993). A yeast protein similar to bacterial two-component regulators. Science **262,** 566-569.

Ouaked, F., Rozhon, W., Lecourieux, D., and Hirt, H. (2003). A MAPK pathway mediates ethylene signaling in plants. EMBO J. **22,** 1282-1288.

Parkinson, J.S., and Kofoid, E.C. (1992). Communication modules in bacterial signaling proteins. Annu. Rev. Genet. **26,** 71-112.

Perrin, D.D., and Sayce, I.G. (1967). Computer calculation of equilibrium concentrations in mixtures of metal ions and complexing species. Talanta **14,** 833-842.

Popov, K.M., Kedishvili, N.Y., Zhao, Y., Shimomura, Y., Crabb, D.W., and Harris, R.A. (1993). Primary structure of pyruvate dehydrogenase kinase establishes a new family of eukaryotic protein kinases. J. Biol. Chem. **268,** 26602-26606.

Popov, K.M., Zhao, Y., Shimomura, Y., Kuntz, M.J., and Harris, R.A. (1992). Branched-chain alpha-ketoacid dehydrogenase kinase. Molecular cloning, expression, and sequence similarity with histidine protein kinases. J. Biol. Chem. **267,** 13127-13130.

Porat, R., Borochov, A., and Halevy, A.H. (1994). Pollination-induced senescence in Phalaenopsis petals--relationship of ethylene sensitivity to activity of GTP-binding proteins and protein-phosphorylation. Physiol. Plant. **90,** 679-684.

Posas, F., and Saito, H. (1998). Activation of the yeast SSK2 MAP kinase kinase kinase by the SSK1 two-component response regulator. EMBO J. **17,** 1385-1394.

Prestridge, D.S. (1991). SIGNAL SCAN: a computer program that scans DNA sequences for eukaryotic transcriptional elements. Comput. Appl. Biosci. **7,** 203-206.

Prestridge, D.S. (1995). Predicting Pol II promoter sequences using transcription factor binding sites. J. Mol. Biol. **249,** 923-932.

Putnam-Evans, C.L., Harmon, A.C., and Cormier, M.J. (1990). Purification and characterization of a novel calcium-dependent protein kinase from soybean. Biochemistry **29,** 2488-2495.

Riechmann, J.L., and Meyerowitz, E.M. (1998). The AP2/EREBP family of plant transcription factors. Biol. Chem. **379,** 633-646.

Rodriguez, F.I., Esch, J.J., Hall, A.E., Binder, B.M., Schaller, G.E., and Bleecker, A.B. (1999). A copper cofactor for the ethylene receptor ETR1 from Arabidopsis. Science **283,** 996-998.

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

Ross, J., and O'Neill, D. (2001). New interactions between classical plant hormones. Trends Plant Sci. **6,** 2-4.

Sakai, H., Hua, J., Chen, Q.H.G., Chang, C.R., Medrano, L.J., Bleecker, A.B., and Meyerowitz, E.M. (1998). ETR2 is an ETR1-like gene involved in ethylene signaling in Arabidopsis. Proc. Natl. Acad. Sci. U. S. A. **95,** 5812-5817.

Satoh, S., and Esashi, Y. (1986). Inactivation of 1-aminocyclopropane-1-carboxylic acid synthase of etiolated mung bean hypocotyl segments by its substrate, S-adenosyl-L-methionine. Plant Cell Physiol. **27,** 285-291.

Satoh, S., Mori, H., and Imaseki, H. (1993). Monomeric and dimeric forms and the mechanism-based inactivation of 1-aminocyclopropane-1-carboxylate synthase. Plant Cell Physiol. **34,** 753-760.

Schaller, G.E., and Bleecker, A.B. (1995). Ethylene-binding sites generated in yeast expressing the Arabidopsis ETR1 gene. Science **270,** 1809-1811.

Schaller, G.E., Ladd, A.N., Lanahan, M.B., Spanbauer, J.M., and Bleecker, A.B. (1995). The ethylene response mediator ETR1 from Arabidopsis forms a disulfide-linked dimer. J. Biol. Chem. **270,** 12526-12530.

Schneider-Poetsch, H.A., Braun, B., Marx, S., and Schaumburg, A. (1991). Phytochromes and bacterial sensor proteins are related by structural and functional homologies. Hypothesis on phytochrome-mediated signal-transduction. FEBS Lett. **281,** 245-249.

Sharrock, R.A., and Quail, P.H. (1989). Novel phytochrome sequences in Arabidopsis thaliana--structure, evolution, and differential expression of a plant regulatory photoreceptor family. Genes Dev. **3,** 1745-1757.

Sherman, M.Y., and Goldberg, A.L. (1991). Formation in vitro of complexes between an abnormal fusion protein and the heat shock proteins from Escherichia coli and yeast mitochondria. J. Bacteriol. **173,** 7249-7256.

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

Stepanova, A.N., and Ecker, J.R. (2000). Ethylene signaling: from mutants to molecules. Curr. Opin. Plant. Biol. **3,** 353-360.

Stock, A.M., Robinson, V.L., and Goudreau, P.N. (2000). Two-component signal transduction. Annu. Rev. Biochem. **69,** 183-215.

Tatsuki, M., and Mori, H. (2001). Phosphorylation of tomato 1-aminocyclopropane-1-carboxylic acid synthase, LE-ACS2, at the C-terminal region. J. Biol. Chem. **276,** 28051-28057.

Thelen, J.J., Miernyk, J.A., and Randall, D.D. (2000). Pyruvate dehydrogenase kinase from Arabidopsis thaliana: a protein histidine kinase that phosphorylates serine residues. Biochem. J. **349,** 195-201.

Tieman, D.M., Ciardi, J.A., Taylor, M.G., and Klee, H.J. (2001). Members of the tomato LeEIL (EIN3-like) gene family are functionally redundant and regulate ethylene responses throughout plant development. Plant J. **26,** 47-58.

Tieman, D.M., and Klee, H.J. (1999). Differential expression of two novel members of the tomato ethylene-receptor family. Plant Physiol. **120,** 165-172.

Tieman, D.M., Taylor, M.G., Ciardi, J.A., and Klee, H.J. (2000). The tomato ethylene receptors NR and LeETR4 are negative regulators of ethylene response and exhibit functional compensation within a multigene family. Proc. Natl. Acad. Sci. U. S. A. **97,** 5663-5668.

Tuazon, P.T., Chinwah, M., and Traugh, J.A. (1998). Autophosphorylation and protein kinase activity of p21-activated protein kinase gamma-PAK are differentially affected by magnesium and manganese. Biochemistry **37,** 17024-17029.

Tuganova, A., Yoder, M.D., and Popov, K.M. (2001). An essential role of Glu-243 and His-239 in the phosphotransfer reaction catalyzed by pyruvate dehydrogenase kinase. J. Biol. Chem. **276,** 17994-17999.

Urao, T., Miyata, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2000). Possible His to Asp phosphorelay signaling in an Arabidopsis two-component system. FEBS Lett. **478,** 227-232.

Urao, T., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2001). Plant histidine kinases: an emerging picture of two-component signal transduction in hormone and environmental responses. Sci. STKE **2001,** RE18.

Vogel, J.P., Woeste, K.E., Theologis, A., and Kieber, J.J. (1998). Recessive and dominant mutations in the ethylene biosynthetic gene ACS5 of Arabidopsis confer cytokinin insensitivity and ethylene overproduction, respectively. Proc. Natl. Acad. Sci. U. S. A. **95,** 4766-4771.

Vriezen, W.H., van Rijn, C.P., Voesenek, L.A., and Mariani, C. (1997). A homolog of the Arabidopsis thaliana ERS gene is actively regulated in Rumex palustris upon flooding. Plant J. **11,** 1265-1271.

Wang, K.L., Li, H., and Ecker, J.R. (2002). Ethylene biosynthesis and signaling networks. Plant Cell **14 Suppl,** S131-151.

Wang, W., Hall, A.E., O'Malley, R., and Bleecker, A.B. (2003). Canonical histidine kinase activity of the transmitter domain of the ETR1 ethylene receptor from Arabidopsis is not required for signal transmission. Proc. Natl. Acad. Sci. U. S. A. **100,** 352-357.

Weyers, J.D.B., and Paterson, N.W. (2001). Plant hormones and the control of physiological processes. New Phytol. **152,** 375-407.

Wilkinson, J.Q., Lanahan, M.B., Yen, H.C., Giovannoni, J.J., and Klee, H.J. (1995). An ethylene-inducible component of signal transduction encoded by never-ripe. Science **270,** 1807-1809.

Xie, C., Zhang, J.S., Zhou, H.L., Li, J., Zhang, Z.G., Wang, D.W., and Chen, S.Y. (2003). Serine/threonine kinase activity in the putative histidine kinase-like ethylene receptor NTHK1 from tobacco. Plant J. **33,** 385-393.

Yanagisawa, S., Yoo, S.D., and Sheen, J. (2003). Differential regulation of EIN3 stability by glucose and ethylene signalling in plants. Nature **425,** 521-525.

Yang, Y., Shah, J., and Klessig, D.F. (1997). Signal perception and transduction in plant defense responses. Genes. Dev. **11,** 1621-1639.

Yeh, K.C., and Lagarias, J.C. (1998). Eukaryotic phytochromes: light-regulated serine/threonine protein kinases with histidine kinase ancestry. Proc. Natl. Acad. Sci. U. S. A. **95,** 13976-13981.

Yen, H.C., Lee, S., Tanksley, S.D., Lanahan, M.B., Klee, H.J., and Giovannoni, J.J. (1995). The tomato Never-ripe locus regulates ethylene-inducible gene expression and is linked to a homolog of the Arabidopsis ETR1 gene. Plant Physiol. **107,** 1343-1353.

Zhang, X.S., and O'Neill, S.D. (1993). Ovary and gametophyte development are coordinately regulated by auxin and ethylene following pollination. Plant Cell **5,** 403-418.

Zhao, X.C., Qu, X., Mathews, D.E., and Schaller, G.E. (2002). Effect of ethylene pathway mutations upon expression of the ethylene receptor ETR1 from Arabidopsis. Plant Physiol. **130,** 1983-1991.

BIOGRAPHICAL SKETCH

Patricia Moussatche was born in Rio de Janeiro, Brazil, in 1975. She demonstrated an interest in science at an early age and started her laboratory training while still in high school. She started her undergraduate studies at the Federal University of Rio de Janeiro in 1994, majoring in biomedicine. In 1996 she moved to the United States as a Distinguished Science Scholar at Bard College, NY. While at Bard, Patricia did two summer internships at the U. of Florida, under the supervision of Dr. Harry Klee. After graduating with a B.A. in biology in 1998, and a brief internship at the Boyce Thompson Institute at Cornell University, she enrolled at the U. of Florida as a graduate student in the Plant Molecular and Cellular Biology Program. The work detailed in this dissertation was conducted in the laboratory of Dr. Harry Klee.