UNIVERSITY OF CALIFORNIA, SAN DIEGO

Characterization of *ETHYLENE INSENSITIVE SIX* and the *ENHANCER OF ETHYLENE INSENSITIVE* in *Arabidopsis thaliana*

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in

Biology

by

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2007

The Dissertation of Ramlah Bliss Nehring is approved, and it is acceptable in quality and form for publication on microfilm:

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University of California, San Diego

2007

DEDICATION

I would like to dedicate my thesis to my loving family and supportive friends. There are friends and then there are friends who answer the phone at 3am - Kat Cosmpolous, Jenn Stubbs, Colleen Noviello, Val Vitkauskas, Tasha Bengoechea and of course my favorite sister in the whole world Grace Nehring, these girls have kept me smiling and kept me sane!

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ACKNOWLEDGEMENTS

I would like to start by thanking Joe Ecker for being my mentor over this rollercoaster that it has been my Ph.D. thesis work. Joe's love of science is infectious, which is definitely a positive for a graduate student mentor. When he gets excited about your project you just can't help but be excited too. He has taught me a lot about independence in science and taking responsibility for my own work. My committee members Joanne Chory, Marty Yanofsky, Bill McGinnis and Tony Wynshaw-Boris have also been crucial to my graduate studies. Thank you so much for finding your way through the maze of the Salk to each of my meetings and giving me so much support and feedback at each of those meetings. I'd like to extend a special thanks to Marty for his aid in helping me find a place in Joe's lab when I was floundering on campus and for being there every time I have turned to him for help since then. Also I couldn't have gotten to where I am now without my pre-UCSD mentor, Ken Feldmann, who encouraged me in applying to graduate schools and has answered many of my questions about science and life along the way.

The Salk Institute's Department of Plant Biology is a wonderful collaborative environment for graduate study. I have depended heavily on the expertise and suggestions of my PBIO colleagues throughout the years in the Ecker lab. Anna Stepanova helped me with transitioning into this project when I joined the lab. Hai Li was one of the best teachers I have ever had; I am so grateful that she was in the Ecker lab for so much of my time there. She provided many invaluable suggestions and was

always available for scientific discussion and was an absolute pleasure to work with. I would also like to thank Ecker lab members Kevin Wang, Sarah Liljegren and Katherine Chang for their help, support and discussions. Having Kim Emerson in the department as our administrative assistant has been wonderful, she is extremely knowledgeable and helpful at all times. And finally I couldn't have made it through my last years in the lab without the assistance of my lab monkeys extraordinaire, including Arun Ganti, Cammie Phelps, Mark Urich and the master of PCR genotyping, illegally dancing in the lab and Jeff Long jokes, Justin Arnold.

The division of Biological Sciences at the University of California San Diego, formerly known as the department of Biology when I joined the program, has an exceptionally well organized graduate program and extremely responsive administrative staff. The funds that they have allocated for students traveling to scientific meetings were vital in my ability to expand my horizons, network with other scientists and assisted me greatly in finding a lab for my postdoctoral research. Additionally I am forever indebted to Tom Tomp for having an open door, always listening to the student's input, answering a million ridiculous questions I've had over the years and assisting with solving graduate student problems no matter how big or how small. He was my very first contact with the biology department and I appreciated his help from day one.

I would also like to extend my thanks to the various funding sources that have supported me while in graduate school at UCSD, first and foremost – Joe Ecker whose

grants paid the bulk of my extended stay at UCSD, additionally the UCSD Genetics Training Grant, Leghler Benbough Foundation and the Chapman Charitable Trust.

I am pleased to say that my life in San Diego has extended far beyond the lab bench. I have made some amazing friends in San Diego and have had some marvelous life experiences during my time at UCSD. Thank you to James Cregg for being my social coordinator, to Ryan Hibbs for cooking me piggy for dinner, to Jennifer Stubbs for being my heterosexual life partner, to Colleen Noviello for all the laughs and runs to Golden Spoon, to Ravi for Ravidon- only the best holiday of the year, to the tri and cycling kids and Sergio for kicking my butt, to Nick Reeves and Laura Erker for igniting my athletic competitiveness, and of course to my entering class – Tom, Will, Jeanne, Nick, Adrienne, Omar, Tasha, TJ, Chris and friends for all the fun, I still miss dessert night! Who knew inner tube water polo could actually be fun? I have missed my family immensely, being on the other side of the country from them all these years. I have loved every single picture that my Papa has sent me over the years; they have kept me feeling so connected to New Hampshire while I haven't been there!

The Introduction, Chapter One, is modified from a book chapter in which I am primary author.

Nehring RB and Ecker JR (2005). Ethylene signal transduction in stem elongation. In: Plant Hormones - Biosynthesis, Signal Transduction, Action! Springer (PJ Davies ed.) pp 350-368.

Chapter Two, in full, consists of the following manuscript in preparation in the format of the journal *Plant Physiology*

Nehring, R. B., McGrath, R. B., and Ecker J. R. *ethylene insensitive six* is Involved in Ethylene Mediated Cytoskeletal Rearrangements and Encodes a Possible Plant Histone Demethylase .

I was the primary researcher and author and Joseph R. Ecker directed and supervised the research that forms the basis for this chapter.

Chapter three consists of other projects I have undertaken during my thesis research, I was the primary researcher on these projects and Joseph R. Ecker directed and supervised the research that forms the basis for these chapters.

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Nehring RB and Ecker JR (2005). Ethylene signal transduction in stem elongation. In: Plant Hormones - Biosynthesis, Signal Transduction, Action! Springer (PJ Davies ed.) pp 350-368.

Maloof JN, Borevitz JO, Dabi T, Lutes J, **Nehring RB**, Redfern JL, Trainer GT, Wilson JM, Asami T, Berry CC, Weigel D, Chory J. Natural variation in light sensitivity of Arabidopsis. Nature Genetics. 2001 Dec;29(4):441-6.

PRESENTATIONS/MEETINGS

Nehring RB and Ecker JR (2006, July) *Analysis of the Transcription Factor ETHYLENE INSENSITIVE6 and the ENHANCER OF ETHYLENE INSENSITIVITY*. Gordon Research Conference, Plant Molecular Biology

Nehring RB and Ecker JR (2005, July) *Molecular and Genetic Analysis of the Transcription Factor ETHYLENE INSENSITIVE6.* Phytochemical Society of North America Annual Meeting

Nehring RB and Ecker JR (2005, June) *Molecular and Genetic Analysis of the Transcription Factor ETHYLENE INSENSITIVE6.* 16th International Conference on Arabidopsis Research

Invited Speaker: *ETHYLENE INSENSITIVE 6 and the ENHANCER OF ETHYLENE INSENSITIVITY*, San Diego Center for Molecular Agriculture, 2005

Nehring RB, McGrath RB, Alonso JM, Ecker JR. (2004, July) *Cloning and Characterization of EIN6 and the EEN.* Gordon Research Conference, Plant Molecular Biology

Characterization of ETHYLENE INSENSITIVE 6 and the ENHANCER OF ETHYLENE INSENSITIVITY, Salk Institute Society of Research Fellows, Presentation and Speaker Award, 2004

Nehring RB, McGrath RB, Alonso JM, Ecker JR. (2003, June) Analysis of *ETHYLENE INSENSITIVE6* and the *ENHANCER OF ETHYLENE INSENSITIVITY*. 14th International Conference on Arabidopsis Research

Nehring RB, McGrath RB, Alonso JM, Ecker JR. (2002, June) Analysis of *ETHYLENE INSENSITIVE6* and the *ENHANCER OF ETHYLENE INSENSITIVITY.* 13th International Conference on Arabidopsis Research

Nehring RB, McGrath RB, Ecker JR. (2001, June) *Molecular Genetic Analysis of Arabidopsis ETHYLENE INSENSITIVE6*. 12th International Conference on Arabidopsis Research

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ABSTRACT OF THE DISSERTATION

Characterization of *ETHYLENE INSENSITIVE SIX* and the *ENHANCER OF ETHYLENE INSENSITIVE* in *Arabidopsis thaliana*

by

Ramlah Bliss Nehring

Doctor of Philosophy in Biology

University of California, San Diego, 2007

Professor Joseph R. Ecker, Chair

Ethylene is a gaseous plant hormone that affects diverse physiological processes throughout plant growth and development. These processes include seed germination, cell elongation, flower and leaf senescence, abscission, sex determination and fruit ripening. Its biosynthesis is mediated by the plants responses to both biotic and abiotic stresses such as pathogen attack, wounding, hypoxia, ozone, chilling and freezing. Molecular genetic studies in *Arabidopsis thaliana* have exploited the triple response to ethylene, and resulted in the identification of mutants defective in this response. After nearly two decades of work in *Arabidopsis*, this model for ethylene signaling has evolved into one of the best characterized signal transduction pathways of all plant hormones. Despite the breadth of knowledge in this field it is still

unknown how many aspects of this pathway function. Additionally new components related to ethylene signaling continue to be discovered. In this work we describe two novel ethylene mutants that act together to cause an ethylene insensitive seedling phenotype. *ETHYLENE INSENSITIVE6* (*EIN6*) when mutated causes an ethylene insensitive root phenotype in *Arabidopsis* etiolated seedlings. The protein encoded by this gene shares homology with Jumonji domain containing proteins that have recently been shown to act as histone demethylases in other eukaryotic organisms. *ein6* mutants also display a pleiotropic range of microtubule related phenotypes not previously documented for other ethylene insensitive mutants. Mutants in the gene *ENHANCER OF ETHYLENE INSENSITIVITY* (*EEN*) do not demonstrate an obvious phenotype on their own, but enhance the ethylene insensitive root phenotype of *ein6* plants to a full ethylene insensitive etiolated seedling phenotype. This is the first enhancer mutation found in the ethylene signal transduction pathway. This study sheds light on two uncharacterized components of the ethylene signal transduction pathway that may be associated with epigenetic control of transcriptional regulation by modification of core histones.

CHAPTER I

INTRODUCTION

ETHYLENE AS A PLANT HORMONE

The gaseous plant hormone ethylene is an olefin hydrocarbon produced by all plants. Despite its simple chemical structure it orchestrates a myriad of complex functions. Ethylene controls processes as diverse as germination, root hair development, root nodulation, senescence of organs (including fruit ripening), differential cell growth, abscission, stress responses and resistance to necrotrophic pathogens (Abeles et al., 1992). It is known that ethylene is effective in inducing a biological response at nanomolar concentrations and that its response takes only minutes to be induced. Due to the important nature of its signaling the production of this hormone is a tightly regulated process controlled by both developmental signals and response to environmental stimuli. To further the understanding of ethylene signaling in plants we need to fully comprehend how the hormone is synthesized and perceived and how the signal is transduced.

ETHYLENE IN SEEDLINGS: THE TRIPLE RESPONSE

Exposure to ethylene induces dramatic morphological changes in the growth of seedlings. Normally an etiolated (dark-grown) seedling displays a closed apical hook, a long slender hypocotyl and an elongated root. When exposed to ethylene seedlings exhibit exaggerated curvature of the apical hook, radial swelling of the hypocotyl, and inhibition of hypocotyl and root elongation (Guzman and Ecker, 1990). This change associated with exogenously applied ethylene was first discovered over 100 years ago in pea seedlings and was termed the “triple response” (Neljubow, 1901). In this context, ethylene is a stress-induced hormone with the triple response mimicking the

natural response that is produced when seedlings encounter a physical barrier as they attempt to penetrate the soil. The exaggeration of the apical hook, due to the production of ethylene, protects the delicate apical meristem from the physical damage that may occur during seedling emergence (Darwin and Darwin, 1881).

Plant biologists have exploited the highly reproducible triple response phenotype in Arabidopsis to discover mutants that affect components in the ethylene signal transduction pathway (Fig. 1). Three main classes of mutants defective in the ethylene response have been identified (Ecker, 1995). These classes are: the ethylene insensitive (*Ein*$^-$) mutants, tissue specific mutants (such as a non-responsive apical hook or root) and "constitutive" mutants that demonstrate a triple response without the addition of exogenous ethylene. The last category can be further divided into two groups: ethylene overproducers (*Eto*$^-$) and constitutive signaling pathway mutants (*Ctr*$^-$).

ETHYLENE PRODUCTION: REGULATION OF BIOSYNTHESIS

In higher plants ethylene is biosynthesized from its *in vivo* precursor, methionine, via the Yang cycle (reviewed by (Yang and Hofmann, 1984)). The Yang cycle requires ATP and produces intermediates S-adenosylmethionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC). The enzymes involved in this pathway are SAM synthetase (SAMS), ACC synthetase (ACS) and ACC oxidase (ACO). Much is known about the ACS enzyme as is detailed below. SAMS catalyzes the biosynthesis of SAM, part of a two gene family in *Arabidopsis*. In tomato the family consists of three genes which are highly expressed and induced by exogenous ethylene

in Arabidopsis (Whittaker et al., 1997). The enzymatic reaction catalyzed by SAMS is not a rate limiting step in the synthesis of ethylene (Yang and Hofmann, 1984). ACO is a ferrous dependent non-heme oxygenase enzyme requiring ferrous iron as a co-factor and oxygen as a co-substrate (Hamilton et al., 1991) that belongs to a small gene family of three to four members in most plants. Ethylene enhances its own biosynthesis by positive feedback regulation of the ACO genes (Petruzzelli et al., 2000). The ACO protein is present at high levels in plant tissues but is very tightly regulated and has a half-life of only 20 minutes *in vitro* (Barlow et al., 1997).

The ACS enzyme is part of a large gene family with 9 members in *Arabidopsis* and 10 members in tomato. These genes can be separated into four classes (Fluhr and Mattoo, 1996) and three evolutionary phylogenetic branches. They have a differential expression pattern through tissues of the developing and adult plant and different isoenzyme diversity among cells and tissues within the plant (Tsuchisaka and Theologis, 2004, 2004). The ACS enzyme is a pyridoxal-5'-phosphate dependent enzyme that converts SAM to ACC and is the rate limiting step in ethylene biosynthesis (Yang and Hofmann, 1984). ACS activity is regulated at both the transcriptional and posttranscriptional levels (reviewed by (Chae and Kieber, 2005). One of the regulatory mechanisms of controlling ACS levels is through phosphorylation of the protein. It was recently discovered that AtMPK6, originally thought to be a downstream component in the ethylene signaling pathway, phosphorylates ACS 2/6, making ACS 2/6 the first characterized plant MAP kinase substrate (Ecker, 2004; Liu and Zhang, 2004). This phosphorylation leads to an

accumulation of the ACS 4/6 and an increase in ethylene production in the plant. AtMPK6 and its tobacco ortholog, SIPK, are quickly activated by external stress on the plant and induce ethylene production. If a plant is mutant for AtMPK6, ACS 2/6 proteins are rapidly degraded leading the conclusion that phosphorylation of the ACS protein leads to a decrease or slowing of proteosome mediated protein degradation. Another ACS class, containing ACS5 and ACS9, has been found to be regulated by its interactions with the protein ETO1 (Wang et al., 2004). The Eto^- (ethylene overproducing) mutants *eto2* and *eto3*, both being dominant mutations found in the c-terminal end of the genes ACS5 and ACS9 respectively, led to the conclusion that the c-terminal end of the ACS proteins are important in regulation of protein stability ((Vogel et al., 1998; Woeste et al., 1999; Chae et al., 2003). This conclusion was further reinforced by the discovery that the interaction of ACS5, and its orthologs from tomato, with ETO1 directly inhibit enzyme activity and target ACS proteins for degradation by a proteosome mediated pathway (Wang et al., 2004). This interaction requires a TOE (target of ETO1) sequence to be present in the ACS protein, making the interaction with ETO1 specific to type II ACS enzymes in *Arabidopsis* and tomato (Yoshida et al., 2006). The interaction between ETO1 and type II ACS enzymes is thought to require a protein phosphorylation event to target the proteins for degradation, but ACS 5 is not phosphorylated although it is the only known protein kinase associated with ethylene biosynthesis, MAPK6, *in vitro* (Liu and Zhang, 2004). Perhaps the phosphorylation of other ACS enzymes is mediated by the calcium

dependant protein kinase (CDPK) found in extracts of wound induced tomato fruits (Tatsuki and Mori, 2001).

THE ETHYLENE SIGNAL TRANSDUCTION PATHWAY

Ethylene Perception via Receptors

A family of five membrane-localized receptors perceives ethylene in Arabidopsis (Fig. 2). The receptors share homology with two-component histidine (His) kinases, originally discovered in bacteria that are involved with sensing environmental changes. Two component systems normally consist of a membrane localized "sensor" protein kinase which detects the input signal and a "response regulator" that mediates the output (Hwang et al., 2002). In plants this family consists of mostly hybrid kinases which contain both the kinase and receptor domains and can be involved in phosphorelay activities, passing along a single phosphate from the receptor through an intermediate phosphorelay protein and finally onto the response regulator (Hall et al., 2000). The ethylene receptors are one of four families of His protein kinases found in plants (Hwang et al., 2002) further divided into two subfamilies (Bleecker, 1999). All of the ethylene receptors contain predicted amino terminal transmembrane domains, and for several of the receptors this domain has been found to contain the site for ethylene binding (Schaller and Bleecker, 1995). ETR1 and ERS1 make up the first subfamily; these two proteins have complete conservation of the His kinase protein motifs and each has three predicted amino terminal membrane spanning domains (Fig. 2). The other three receptors (ERS2, EIN4 and ETR2) are part of the second subfamily, characterized by a signal sequence

whose putative function may be to target proteins to the secretory pathway. This subfamily lacks some of the amino acids previously shown to be essential for His kinase activity, casting some doubt on the designation that these are functional His kinases, but they may act as serine/threonine kinases (Moussatche and Klee, 2004). All members of this subfamily contain four n-terminal hydrophobic stretches that may act as membrane spanning domains. The n-terminus of the receptor proteins contains the domain responsible for the actual binding of the ethylene molecule (O'Malley et al., 2005). In this study it was found that there is a direct correlation between total message level, RNA transcript level of all receptors, and total ethylene binding capacity. When one or more of the receptors is mutated, the level of the functional receptors mRNA can increase to compensate for the mutant isoforms. This compensation allows for the same level of ethylene binding but not the same level of signaling. The receptors, members of both family one and family two, release ethylene with the same slow dissociation kinetics. Previously it had been thought that there were both fast-release and slow-release binding kinetics found in the dissociation of ethylene from its receptors (Hall et al., 1990; Sisler, 1991). Previous to this finding about compensation at the RNA level it had also been postulated that loss of one receptor might be compensated by up-regulation of another receptor's protein level. This was not shown to be the case with ETR1, because in the mutant backgrounds *etr2-3, ein4-4*, and *ers2-3* (including combinations of single, double and triple mutants) the level of ETR1 protein was similar to wild type levels (Zhao et al., 2002).

The receptors have been hypothesized to require a transition metal in order to bind the olefin ethylene (Burg and Burg, 1967). Based upon the *in vitro* biochemical association of copper ions and the binding domain of ETR1 (Rodriguez et al., 1999), and the *in planta* identification of RAN1, a copper-transporter, the metal in the ethylene receptors has been revealed to be copper. Defects in RAN1 cause altered ligand specificity of the ethylene receptors and reduced function of the entire ethylene pathway (Hall and Bleecker, 2003). Silver ions, similar to copper in their capability to interact with ethylene, cause an inhibitory effect on all plant responses to ethylene phenocopying the ethylene insensitive mutants (Bleecker and Kende, 2000). Another gene, *REVERSION-TO-ETHYLENE SENSITIVITIY*, *RTE*, was recently found to act at the same level as the receptors (Resnick et al., 2006). Alleles of *rte* were found to be suppressors of the weakly ethylene insensitive mutant *etr1-2*, and *rte* mutants alone have an enhanced ethylene response phenotype. *RTE* is induced by ethylene gas treatment. The putative protein coding for this gene is evolutionarily conserved, but lacks any known protein motifs and has no previously classified function.

Ethylene is readily diffusible through both aqueous and lipid environments, allowing for one of the receptors, ETR1, to be localized to the endoplasmic reticulum of *Arabidopsis* cells (Chen et al., 2002). The receptors are expressed throughout plant tissues and have overlapping domains of expression. *ETR1* mRNA is down-regulated in the apical hook of etiolated seedlings, while the other receptors are shown to be up-regulated in adult leaves (Alonso and Ecker, 2001). Mutant analysis revealed dominant mutations in the receptors result in ethylene insensitivity (Chang and

Stadler, 2001). While conversely combinations of recessive receptor mutants result in a constitutive signaling phenotype (Hua and Meyerowitz, 1998). The genetic prediction based on these opposite phenotypes of the loss of function and gain of function mutants suggests that when ethylene is unable to bind to the receptors that they remain active, and that the receptors normally function as negative regulators of the downstream ethylene signal transduction pathway (Hall et al., 2000). Further insight has been made into the differences between the type 1 and type 2 subfamilies of receptors using biochemical approaches and newly discovered null mutations in one of the receptors. The protein level of ETR1 has been found to be up-regulated in the *etr1* ethylene insensitive mutants (*etr1-1*, *etr1-2*, *etr1-3* and *etr1-4*) (Zhao et al., 2002). The ethylene insensitivity of these mutants was equivalent to treating the seedlings with silver; demonstrating that silver mimics an ethylene insensitive mutation. Interestingly, only single null alleles of subfamily I show any phenotypes; a slight increase in ethylene sensitivity is seen in the alleles (Hua and Meyerowitz, 1998). When combining newly identified null alleles of subfamily I; *etr1-9* and *ers1-3*, these plants display a strong constitutive activation of the ethylene pathway (Qu et al., 2007). The response seen from this mutant combination is stronger then any other previously characterized receptor mutant combination, even the quadruple LOF (Hua and Meyerowitz, 1998). The combination of these two alleles has demonstrated that subfamily one plays the predominant role in regulating ethylene responses (Qu et al., 2007). The adult phenotypes observed in the *etr1 ers1* double mutant include small rosette size, delayed flowering, and defects in fertility and flower morphology. These

phenotypes are even more severe than the adult phenotypes of the constitutive ethylene signaling mutant, *ctr1* (Hall and Bleecker, 2003). Whether the pleiotropic defects seen in the double mutant plants were ethylene related or ethylene independent was determined by generating a triple mutant of *ers1-2*, *etr1-7* and *ein2* (Wang et al., 2003). These mutants showed an *ein2*-like ethylene insensitive phenotype and therefore demonstrated that the receptor defects are dependant on EIN2 (Hall and Bleecker, 2003). The double mutant phenotypes in both light and dark grown seedlings could be rescued by either of the wild type cDNAs for *ETR1* or *ERS1*, but not by the cDNAs for the type 2 subfamily of receptors, *EIN4*, *ERS2* or *ETR2* (Wang et al., 2003).

DOWNSTREAM SIGNALING COMPONENTS

The negative regulator of the ethylene pathway CTR1

The first ethylene signaling pathway gene that was cloned in plants was identified as a T-DNA tagged allele of the constitutive ethylene signaling mutant *ctr1* (Kieber et al., 1993). Plants containing loss of function mutations in *CONSTITUTIVE TRIPLE RESPONSE1* look as if they are constantly exposed to ethylene in both seedlings and adults, indicating that this protein acts as a negative regulator of ethylene signaling. The protein encoded by the CTR1 gene shares homology with the mammalian Raf serine/threonine protein kinase family. The role of these proteins in mammals is acting as a mitogen-activated protein kinase kinase kinase (MAPKKK). The similarity of CTR1 to MAPKKKs suggested a downstream MAP kinase cascade in the ethylene pathway (Fig. 3).

The CTR1 protein is a very likely candidate for the next step in the putative phosphorelay cascade proposed for the ethylene receptors. It has been demonstrated to possess *in vitro* Ser/Thr kinase activity. Biochemically, the amino terminus of CTR1 has also been shown to interact directly with the ethylene receptor ETR1 via a putative CTR specific protein-protein interaction domain called the CN box (Huang et al., 2003). These studies suggest that in the absence of ethylene, the ethylene receptors are active and stimulate the kinase activity of CTR1, allowing it to phosphorylate downstream targets and causing the ethylene pathway to be repressed. Weak associations between CTR1 and other ethylene receptors, such as ERS1 and ETR2, have also been recognized (Cancel and Larsen, 2002), implicating both subfamily 1 and 2 of the receptors in binding/localization of CTR1. Interestingly, the histidine kinase activity of ETR1, when expressed in yeast, is not required for the binding of CTR1 to the receptor (Gao et al., 2003). The amino terminal regulatory and the kinase domains within CTR1 have also been found to associate with themselves (Larsen and Cancel, 2003). This might be a mechanism of negative regulation causing an inhibition of CTR1 kinase activity. CTR1 is postulated to bind to the receptors by being associated within the same location in the cell (Gao et al., 2003). CTR1 has been isolated as part of an ethylene receptor signaling complex, including the receptor ETR1. Using sucrose density centrifugation CTR1 was localized to the ER of *Arabidopsis* microsomes (Gao et al., 2003). The ethylene receptors are required for the membrane localization of CTR1, because when multiple

receptors were mutated CTR1 was relocalized to the soluble fraction of the cell (Gao et al., 2003).

Ethylene signaling does not appear to be completely dependant upon CTR1 signaling. Plants containing strong alleles of *ctr1*, which do not have any kinase activity, still respond weakly to ethylene treatment (Kieber et al., 1993). It has also been shown that quadruple ethylene receptor mutants demonstrate a constitutive phenotype that is more severe than the strongest *ctr1* alleles. Additionally the strong *ran1-3* allele, of the copper transporter, also has a more severe ethylene phenotype (Woeste and Kieber, 2000). The "residual" ethylene response observed in *ctr1* plants may be a result of the activity of other CTR1-like proteins that are as of yet undetermined.

The Central Signaling Component EIN2

Loss of function mutations in *EIN2* confers complete plant ethylene insensitivity, indicating that EIN2 is an essential positive regulator of the ethylene signal transduction pathway (Alonso et al., 1999). Over 25 *EIN2* alleles have been discovered, more than of any other ethylene signaling pathway component. In part, this is because *ein2* plants, relative to all other mutants, display the least response to ethylene; they display the greatest degree of ethylene insensitivity. In addition, many alleles of *ein2* have been uncovered in mutant screens for plants with defects in the other signaling pathways including responses to cytokinins, abscisic acid, sugars and in screens for delayed senescence (Wang et al., 2002). The appearance of *EIN2* occurs so recurrently in such a divergent set of mutant screens indicates that this protein plays

a essential role in ethylene signaling and that ethylene plays a role in modulating responses to a diverse set of signals, each of which also shows unique plant responses that are unrelated to ethylene.

EIN2 encodes an integral membrane protein with 12 predicted transmembrane domains at its amino terminus (Alonso et al., 1999). This domain shares homology with the Nramp family of metal-ion transporters. This family is made up of members such as yeast SMf1p, *Drosophila* Malvolio and mammalian DCT1 (Alonso and Ecker, 2001). No studies have been able to identify a metal transporting activity for EIN2, nor is EIN2 able to complement metal uptake deficient yeast strains as shown for authentic *Arabidopsis* Nramp genes (Schaller and Kieber, 2002). The carboxy-terminal region of EIN2 protein does not have homology to other proteins of known function. This hydrophilic region contains domains that may be associated with protein-protein interactions. Interestingly, it is this unknown part of the EIN2 protein that is sufficient to elicit the ethylene response in Arabidopsis plants. Plants overexpressing the unique carboxy-terminal end (C-END) of the protein show a constitutive ethylene response in both light grown seedlings and in adult plants. These plants also constitutively express (at the mRNA level) all ethylene-regulated genes. The overexpression of EIN2 C-END is also sufficient to activate the downstream nuclear pathway through EIN3 (Alonso et al., 1999). However, overexpression of the C-END was not able to induce the triple response in etiolated seedlings, indicating that the Nramp domain must be necessary for ethylene mediated effects in dark grown seedlings. These results suggest that the Nramp domain of EIN2 is required for

sensing the upstream ethylene signal while the carboxy-terminal domain of EIN2 is necessary for transducing the signal to downstream ethylene pathway components.

Nuclear Events: The EIN3 Family and its Target Genes

The plants' response to ethylene gas is known to involve changes in gene expression (Stepanova and Ecker, 2000). However, until identification of the *ein3* mutant and cloning of the *EIN3* gene there was no direct evidence of nuclear regulation in the ethylene signaling pathway (Chao et al., 1997). Mutations in *ein3* in Arabidopsis cause reduced response to ethylene, although these plants are more sensitive to ethylene than *ein2* mutants. This reduced response is seen in an ethylene insensitive dark grown seedling, reduced ethylene-dependent expression of genes. Additionally adult plants have reduced ethylene-mediated leaf senescence (Chao et al., 1997). This insensitivity phenotype displayed in all loss of function mutations indicates that *EIN3* is a positive regulator of ethylene signal transduction.

The protein encoded by the *EIN3* gene is a member of a family of six related proteins in Arabidopsis. These nuclear-localized transcriptional activators contain an acidic domain at their amino terminus along with a proline-rich region, a coil structure and several regions that contain highly basic amino acids. The amino terminus of these proteins is involved in DNA binding (Solano et al., 1998). Members of this family are closely related in sequence and function; in fact, two of the *EIN3*-like genes (*EILs*), *EIL1* and *EIL2* can rescue the mutant phenotype of *ein3*, indicating that these related proteins must also be involved in ethylene signal transduction. Mutants for *ein3* show only a reduction of function for ethylene signaling, unlike the strong loss of

function mutants such as *ein2* or *etr1*. Possible functional overlap between EIN3 family members might account for this reduced phenotype (Chao et al., 1997). Additionally, overexpression of either *EIN3* or the *EIL1* induces a constitutive ethylene response similar to the phenotype seen in the *ctr1* mutant, demonstrating the sufficiency of these proteins to activate the ethylene response pathway in both seedlings and adult plants (Chao et al., 1997). The *EIN3* overexpression phenotype is independent of the presence of a functional EIN2 protein indicating that the EIN3/EIL family functions downstream of EIN2 in the ethylene pathway. Interestingly, *EIN3* gene expression is not affected by ethylene treatment (Wang et al., 2002). Recent studies have revealed the nature of posttranslational regulation of EIN3 (Guo and Ecker, 2003; Potuschak et al., 2003). EIN3 protein can be stabilized when proteosome function is inhibited, mimicking the effect of exogenous ethylene treatment on regulation of the protein (Guo and Ecker, 2003). Two F box proteins, EBF1 and EBF2 have been found to directly interact with EIN3 and EIL1 and lead to EIN3 (and probably EIL1 as well) proteolysis (Potuschak et al., 2003). Mutants in the genes encoding *EBF1* and *EBF2* result in hypersensitivity to ethylene and an increase in EIN3 protein accumulation. The double mutant *ebf1ebf2* results in *ctr1* like phenotypes, and suppress *ein2,* indicating that these genes function downstream of or parallel to EIN2 in the ethylene signaling pathway (Potuschak et al., 2003). The transcription of the *EBF1* and *EBF2* genes is disrupted in the *ein3* mutant. These studies indicate the presence of a possible negative-feedback mechanism where on one side the production of ethylene acts to stabilize and prevent degradation of the EIN3

protein, on the other side, EBF1 and EBF2 are induced when EIN3 is stabilized and target EIN3 for proteosome mediated degradation. The ubiquitin/proteosome pathways have been demonstrated or implicated in most other plant hormone signaling pathways, leaving us to wonder whether this type of protein regulation contributes to the well-documented cross-talk between various phytohormones. One of the targets of the EIN3/EIL proteins was discovered when an ethylene response element binding protein (EREBP), *ERF1*, was found to require *EIN3* function for its expression (Solano et al., 1998). *ERF1* mRNA was not detectable in *ein3* mutants, and its steady-state levels were dramatically increased in EIN3 overexpressing plants. The amino-terminal end of EIN3 was found to specifically bind to the promoter of the *ERF1* gene. In addition the related proteins EIL1/2 were also able to bind to the EIN3 binding site in the *ERF1* promoter (Solano et al., 1998). The EIN3/EIL proteins were also able to bind to the gene promoter of another unrelated transcription factor EDF1 (Wang et al., 2002). A short palindromic fragment of DNA was identified as the EIN3 binding site. This binding site has been classified as a primary ethylene response element (PERE), and is similar to sequences in promoter regions required for ethylene regulated gene expression in other species. EIN3 binds to this site as a homodimer, and, from *in vitro* DNA binding studies, it does not form heterodimers with its related family members (Solano et al., 1998).

One known target of EIN3, *ERF1*, is a transcription factor itself. This suggests a transcriptional cascade where EIN3 binds to the promoter of *ERF1*, and ERF1 protein in turn regulates other EREBPs (Solano et al., 1998). Using *in vitro* binding

studies, ERF1 has been shown to bind specifically to the promoters of ethylene-regulated genes containing the GCC box. ERF1 is also a positive regulator of ethylene signaling similar to EIN3, and yields a constitutive ethylene phenotype similar to EIN3 when overexpressed in plants. The EREBPs were originally identified because they contained a highly conserved DNA binding domain (the ERF domain) which is able to bind to the GCC box, a *cis*-element found in the promoters of many ethylene responsive pathogen-related genes. Many *EREBP* genes exist in Arabidopsis and other monocots and dicots (Riechmann and Meyerowitz, 1998), but only a few of these related genes are directly regulated by ethylene. These genes are, however, also regulated by various abiotic stress responses such as those induced by cold, drought, salt, wounding and pathogens. The EREBPs can function as either transcriptional activators or transcriptional repressors (Fujimoto et al., 2000). As transcriptional repressors, they are active repressors suppressing the transactivation of other transcription factors without competing for the same DNA binding site.

Other Ethylene Response Mutants

Numerous other ethylene mutants have been identified, both through triple response screens as well as other mutant screening approaches. Three other alleles of ethylene insensitive mutants have yet to be characterized (*ein5*, *ein6* and *ein7*) (Roman et al., 1995). All of these alleles show a reduced response to ethylene, but do not display as severe ethylene insensitive when compared with *ein2*. Using double mutant analysis, these ethylene insensitive mutants are found to be epistatic to CTR1. Two recessive alleles of *ein5*, along with one semi-dominant allele of *ein7* were originally

discovered, *ein7* was found to be another allele of *ein5* upon sequencing (Olmedo et al., 2006). *EIN5* was recently found to be an allele of a 5' to 3' exoribonuclease, XRN4 (Olmedo et al., 2006; Potuschak et al., 2006). *XRN4* has been identified as the *Arabidopsis* homolog of the budding yeast XRN1 protein (Souret et al., 2004). The protein appears to act on the ethylene signaling pathway by regulating the F-box genes *EBF1/2* mRNA levels and therefore affecting the proteosome mediated turnover of EIN3. *ein5* mutants have increased amounts of *EBF1/2* transcript and therefore lower levels of EIN3 protein causing a dramatic decrease in downstream ethylene signaling.

The *ein6* mutant was originally characterized as a recessive mutation with reduced gametophytic transmission. The ethylene insensitive phenotype of this mutant has now been attributed to not one but two mutations in the background (Nehring and Ecker unpublished). The double mutant resulting in the *ein6* phenotype has a number of other interesting phenotypes such as taxol hypersensitivity (Roman and Ecker, 1995). The taxol result is interesting because cortical microtubule reorientation, from transverse to longitudinal, has been implicated as a downstream effect of the ethylene pathway. Since only single alleles were generated for these mutants perhaps screening for triple response mutants has not been yet saturated.

Altering the triple response assay has found a number of other new ethylene mutants. One of these mutants was found in a screen for plants that had enhanced ethylene responsiveness (EER). Compared to wild type, Eer^{-} plants displayed greater sensitivity and increased amplitude of the response to ethylene. One mutant isolated from this screen, *eer1*, has a short thick hypocotyl in response to treatment with ACC,

ethylene gas and also in response to ethylene antagonists (Larsen and Chang, 2001). The *eer1* hypocotyls are only about 50% the length of wild-type seedlings in the presence of exogenous ethylene. This phenotype was not caused by a constitutive signaling defect, due to the fact that it can be reversed by treatment with an inhibitor of ethylene biosynthesis (AVG). In combination with other known ethylene mutants *eer1* showed a number of interesting phenotypes. Many of these were suppressed by the ethylene insensitive mutants, *ein2* and *etr1*, but several phenotypes showed an additive effect when combined with *ctr1*. The mutant also showed an increase in expression of a known ethylene response reporter gene, *BASIC-CHITINASE*. Taken together, these studies suggest that the phenotype of this mutant is not simply the result of ethylene overproduction, but these plants may be highly sensitive to endogenous ethylene. Recent cloning of *EER1* revealed that it encodes a previously described Arabidopsis PP2A regulatory subunit, also known as *RCN1* (Larsen and Cancel, 2003). Placing EER1 in the ethylene pathway is difficult due to the complex phenotypes. It has been proposed to be partially responsible for the regulation of CTR1, or another related MAPKKK, either through the known ethylene pathway or through an alternative ethylene-signaling pathway (Larsen and Chang, 2001; Larsen and Cancel, 2003). A second mutant with the same enhanced ethylene phenotype, *eer2*, has also been isolated from the same screen but its gene identity is as of yet unknown (De Paepe et al., 2005).

Another variation on the standard triple response assay involves looking for ethylene mutants that are only partially lack the normal response. Using low doses of

ethylene, a number of weakly ethylene-insensitive (Wei$^-$) mutants have been identified (Alonso et al., 2003; Stepanova et al., 2005). Three of the mutants, *wei1*, *wei4* and *wei5*, were found to correspond to previously known genes, *TIR1*, *ERS1* and *EIL1* respectively. The other mutants, *wei2*, *wei3* and *wei7*, are thought to be previously unidentified members of the ethylene response pathway. TIR1/WEI1 is thought to participate as an ubiquitin ligase in the ubiquitin-mediated degradation of auxin response proteins. Uncovering *ers1/wei4* in a mutant screen is the first time that this gene has ever been found in a mutant screen, validating that this assay is uncovering new mutants. Identification of alleles in the ethylene receptors similar to this one may give us insights into the normal developmental context of gene function. The EIL genes had previously been suggested to be involved in the ethylene response (Chao et al., 1997) and the isolation of *eil1/wei5* confirms the involvement of *EIL1* in the ethylene-signaling pathway. The remaining WEI$^-$ mutants, showed mainly root-specific phenotypes. In the past other ethylene insensitive root mutants have been discovered, but these genes were found to actually be related to auxin responses (see the section on ethylene and other growth regulators). Interestingly the WEI$^-$ mutants show normal sensitivity to exogenous auxin and a normal growth response to gravity. Two additional WEI mutants, *wei2* and *wei7* show a root specific ethylene insensitive phenotype. Upon cloning these two genes it was discovered that they are alleles of *ANTHRANILATE SYNTHASE* genes (*ASA1* and *ASB1*) involved in the biosynthesis of endogenous auxin (Stepanova et al., 2005). This finding has shown direct regulation of the formation of auxin molecules by ethylene. The role of *WEI 2/7* is not isolated

only to the ethylene response, but also appears to be important for general auxin biosynthesis as seen by the repression of the high-auxin phenotypes seen in double mutant combinations of either of the *wei2* or *wei7* alleles with the auxin overproducing mutants *superroot1* (*sur1*) or *sur2*.

The number of newly uncovered mutants within the ethylene pathway continues to grow, demonstrating that genetic screens have not been saturated, although new twists on the old standby (triple response) might yield the best results.

OTHER ETHYLENE RESPONSES IN THE SEEDLING

The Response of the Seedling to Light and Ethylene

Ethylene has been shown to dramatically inhibit cell expansion. In the earliest studies on the hormone it was shown that exposing pea seedlings to ethylene caused an inhibition of epicotyl elongation (Neljubow, 1901). Similarly, prominent effects of ethylene in Arabidopsis include short roots and inflorescences, and an overall stunted appearance (Kieber et al., 1993). One more recently described phenotype induced by the hormone is an increase in the length of the hypocotyl of Arabidopsis seedlings grown in the light in the presence of ACC or ethylene (Smalle et al., 1997). Most dramatically the length was increased 2-fold when the seedlings were grown on a nutrient-deficient growth medium. This increase in length is not a result of increased cell division, but instead of an increase in cell elongation and overall cell volume. The increase in hypocotyl length was confirmed to be caused by ethylene treatment because it could not be induced in the ethylene insensitive mutant *etr1,* and was reversed by treatment with the ethylene antagonist, silver. This effect of ethylene

might be employed as a screening method to uncover additional ethylene signaling mutants.

Ethylene and Mechanical Stimuli

Changes in the growth of plants in response to physical environmental forces with which they physically interact are known as thigmomorphogenesis. When plants physically interact with their environment through processes such as rubbing, touching, rain, wind or due to growth against an object, they alter their growth habit to offset these mechanical stimuli (MS). This process is known to be calcium dependent and causes a rapid elevation in the amount of cytosolic free-calcium. Plants up-regulate a specific set of genes known as the touch genes (*TCH*) within a few minutes of receiving a stimulus (Braam and Davis, 1990). While plant hormones/growth regulators may somehow mediate this process, the signal transduction pathway from perception to response is still unknown. In this regard, MS can induce ethylene gas production and ethylene can induce the expression of the gene *TCH3* in the absence of a mechanical stimulus (Sistrunk et al., 1994).

A second connection between ethylene and mechanical stimuli can been seen in seed germination. Ethylene is known to promote seed germination and certain ethylene insensitive mutants (*etr1*) are known to have lower germination rates then wild type (Bleecker et al., 1988). Vibration, a type of MS, is known to promote seed germination as well. Vibration-induced promotion of germination is not seen in the seeds of the Arabidopsis ethylene insensitive mutant *etr1* (Uchida and Yamamoto, 2002). This response was also lacking when wild-type seedlings were treated with the

inhibitor of ethylene production, AVG. This finding indicates that alternate signal transduction pathways in response to MS may be present in the seed and germinated-seedling stages of development.

ETHYENE AND OTHER GROWTH REGULATORS

Ethylene has been found to play a central role in global hormone responses. It controls not only its own response pathway, but has also been found to regulate the synthesis of and response to other hormones and growth regulators.

Ethylene and Auxin

The intersection between the pathways for the biosynthesis and signaling of the hormones ethylene and auxin is known to occur at many levels. Auxin is known to upregulate the expression of ethylene biosynthesis genes, such as the rate-limiting enzyme ACS (Theologis, 1989; Abel et al., 1995). A number of genetic screens for mutants with aberrant responses to ethylene application have yielded mutants in genes required for auxin signaling. One of the earliest discovered was the mutant *HOOKLESS1* (*HLS1*) (Guzman and Ecker, 1990). *hls1* mutants lack a normal differential growth response to ethylene in the apical region of the hypocotyl; these plants do not form an apical hook in ethylene. These mutants also show abnormally enlarged cells in the hypocotyl and cotyledon. This defect can be phenocopied when seedlings are treated with either auxin or auxin-transport inhibitors, suggesting a link between auxin and ethylene signaling. The promoter region of HLS1 contains an ethylene inducible GCC-box, making it a possible target of the EREBPs (Stepanova and Ecker, 2000). The HLS1 protein encodes a putative N-acetyltransferase, which

may function in acetylating a protein involved in auxin transport or signaling (Lehman et al., 1996). The role of HLS1 was further elucidated by undertaking a suppressor screen of the mutant (Li et al., 2004). Three alleles of *HOOKLESS1 SUPRESSOR* (*HSS1*) were isolated in the screen to reverse the *hls1* phenotype in the apical hook of dark grown seedlings and adult plants, this mutant turned out to be allelic to *AUXIN RESPONSE FACTOR2* (*ARF2*). *ARF2* is expressed in the hook region of seedlings, as is *HLS1*. The ARF2 protein accumulation is negatively regulated by HLS1, with an increase in ARF2 seen in the *hls1* mutant. The AFR2 protein level is also downregulated by exogenous ethylene treatment and this downregulation appears to be proteosome mediated. The isolation of *hls1* and its suppressor *hss1/arf2* has allowed insight into the control of differential cell elongation in the apical hook region of seedling. It has been known that signals from ethylene, auxin and light all control the formation of the apical hook, but these studies shed light on how this happens; specifically that differential elongation is mediated by *ARF2* in a *HLS1* dependent manner.

There are a number of other mutants with tissue specific defects in response to ethylene, which are now implicated in auxin signaling. Three mutants, *eir1*, *axr1* and *aux1* all display ethylene insensitivity in the root (Alonso and Ecker, 2001). EIR1 is an auxin efflux carrier, and plants containing mutations in this gene are not resistant to high levels of auxin. Cloning of *AXR1* revealed that it might be involved in the ubiquitin-mediated degradation of a part of the auxin-signaling cascade.

AUX1 and EIR1 were found to be proteins which regulate polar auxin distribution. AUX1 is an auxin influx carrier, and plants containing mutations in this gene are also resistant to auxin. AUX1 has now been implicated as part of a three gene signal that is required for the planar polarity needed for the correct localization of the emerging root hair (Fischer et al., 2006). Root hairs normally emerge at the basal ends of hair forming cells, and this basal initiation site is hyperpolarized by treatment with auxin or ethylene. Three mutants, *aux1*, *gnom* and *ein2*, are all known to have a change in this polarity resulting in a more root hairs that emerge from the apical ends of cells. When double or triple mutant combinations of these three genes are formed this erratic emergence of root hairs is enhanced. These findings suggest that these genes are involved in establishing the auxin gradient and the vectorial clue for planar root hair positioning.

The mutant *nph4* shows a specific defect in its auxin-mediated response: the hypocotyl of this mutant is unable to bend in response to laterally applied blue-light or after auxin application. The auxin-related phenotype observed in *nph4* is complemented by exogenous application of ethylene. Interestingly, this ethylene effect is blocked in the *hls*1 mutant, demonstrating a synthetic requirement of HLS1 for blue-light-mediated (auxin-dependent) differential growth. Because *NPH4* encodes a member of the ARF family of auxin response (transcription) factors, *ARF7*, these results suggest that ethylene compensation of bending in *nph4* mutants may occur by activation of other ARF family members (Alonso and Ecker, 2001). Another family member that is known to be closely involved in similar functions to *ARF7* is its closest

homolog *ARF19* (Li et al., 2006). *arf7* and *arf19* have ethylene insensitive root phenotypes and this phenotype is exaggerated in the double mutant combination *arf7arf19*. *ARF19* can complement the defects of an *arf7* mutant, indicating that *ARF7* and *ARF19* have overlapping functions. *ARF19* is induced by exogenous treatment with either ethylene or IAA. Other *ARF*s may also be related to both ethylene and auxin but because the *ARF* transcription factor family is so large , containing 23 members in Arabidopsis, and members are known to have functional redundancy, it is difficult to isolate the unique role of each family member.

More recently, there have been two additional ethylene-related mutants identified that show "cross-talk" between ethylene and auxin responses. The mutant *eer1* (see the section on Downstream Signaling Pathway Components) was discovered due to its enhanced response to ethylene. Upon cloning the gene it was discovered to be a mutation in the previously characterized gene *RCN1*. Seedlings mutant for *rcn1* show altered auxin transport, specifically increased basipetal auxin transport, due to their reduced phosphatase activity (Rashotte et al., 2001). The effect of ACC or ethylene on light grown seedlings has been exploited to find new mutants with altered responses to ethylene (Smalle et al., 1997). Recently a mutant, *ACC-related long hypocotyl 1* (*alh1*) was isolated in a screen for mutants, which displayed elongated hypocotyls when grown in the light on nutrient-deficient growth medium without the presence of hormone (Vandenbussche et al., 2003). These mutants were found to overproduce ethylene, but were also altered in their response to auxin. It is thought

that this mutant may affect ethylene-auxin cross talk, possibly by regulating the transport of auxin in the hypocotyl.

Ethylene and the Stress Hormones

Responses to ethylene are known to overlap with two other stress signals: jasmonic acid (JA) and salicylic acid (SA). The combinatorial action of these signals regulates plant defense responses to a variety of pathogens.

Ethylene and JA signaling can be interdependent, functioning as both positive and negative regulators of each other to provide the correct pattern of expression of the systemically induced defense-related genes. This interaction is mediated, at least partially, through the gene *ERF1*. Recent findings have indicated that *ERF1* is a common downstream target of both ethylene and JA in response to pathogen attack. This key gene can be induced by exogenous treatment by either hormone. However, the induction of *ERF1*, and its target genes, requires both JA and ethylene pathways to be intact, as seen by lack of *ERF1* gene expression in the ethylene mutant, *ein2*, and the JA mutant, *coi1* (Lorenzo et al., 2003). The induction of downstream defense genes such as P*DF1.2* and *BASIC CHITINASE* (*b-CHI*) also requires that upstream elements of both these pathways are functional (Solano et al., 1998). The induction of downstream target genes can be fully rescued by introduction of *ERF1* expression in the ethylene/JA mutant backgrounds. *ERF1* is also sufficient to confer resistance to necrotrophic fungi such as *B. cinerea* (Berrocal-Lobo et al., 2002). Finally, expression of the *ERF1* gene requires both ethylene and JA, demonstrating the necessity of both pathways in plant resistance to necrotrophic pathogens.

While ethylene and JA act in a synergistic manner to provide plants with resistance to necrotrophic pathogens, SA has been shown (in some instances) to be an antagonist. Overexpression of ERF1 or activation of the ethylene pathway has a detrimental effect on plant resistance to *Pseudomonas syringae tomato* DC3000 (Berrocal-Lobo et al., 2002). There are, however, considerable interactions between these two pathways, or at least the outputs of these two pathways can overlap (or have common targets). With the advent of expression arrays, it has been shown that many genes respond to two or more defense signals when treated with SA, JA, ethylene or pathogen infection (Wang et al., 2002). Positive interactions between ethylene-dependant and SA-dependant pathways mediating disease responses have also been found, such as the necessity for both pathways to be active in the response to the pathogen *Plectosphaerella cucumerina* (Berrocal-Lobo et al., 2002). Interestingly an SA dependant, *NPR1* (non-expresser of *PR-1*) independent pathway has been discovered which requires a loss of function of the ethylene-signaling pathway (for example, a mutation in *ein2*) to fully abolish *PR-1* gene expression (Clarke et al., 2000). This response involves two mutants that have been found to constitutively express *PR* genes, *cpr5* and *cpr6*. The mutant, *ein2*, also controls the level of SA in these mutants, causing accumulation in *cpr5* and decreasing accumulation in *cpr6*. Yet another player has been discovered between SA and JA/ethylene dependent pathways in the suppressor of *npr1*, *ssi1*. This mutant constitutively expresses *PDF1.2* in an SA dependent manner, linking it to both pathways along with CPR5 and CPR6.

CONCLUSIONS

The understanding of the myriad of roles of ethylene in plant development and disease has greatly advanced in the last 20 years. In particular, studies of the effects of ethylene on the development of dark grown seedlings of the reference plant *Arabidopsis thaliana*, have allowed the identification of many mutants in this signaling pathway. In turn, these mutants have lead to identification of many of the components of the ethylene signaling pathway and provided insight into the mechanisms of ethylene regulation of plant growth/development and response to pathogens. However, there are still many more pieces of the ethylene puzzle to uncover. New methods, such as additional novel mutant screens, genome-wide expression studies, the identification of transcription factor binding (EIN3/EIL/ERF1/EREBP) sites in combination with chemical and reverse genetic approaches, will need to be employed to further our understanding of mechanisms of action of this critical plant hormone.

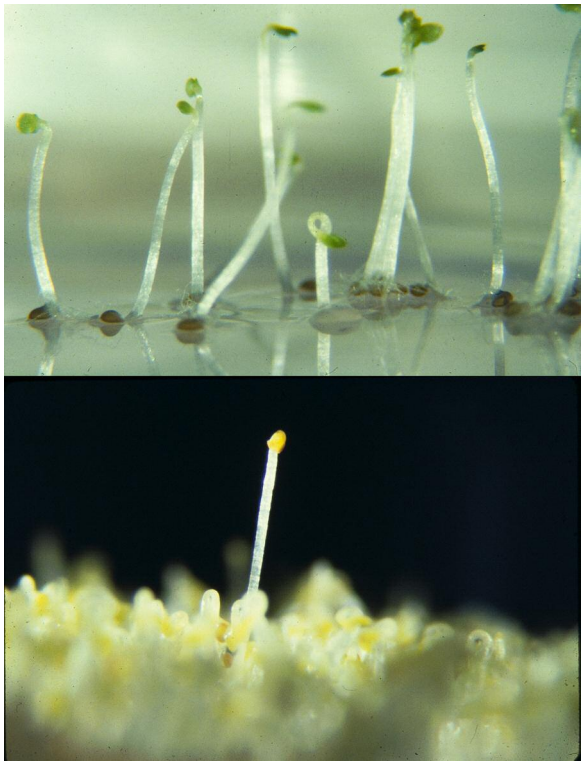


Figure 1.1. The ethylene triple response.

Seedlings were grown in the dark and treated with ethylene gas three days after germination. (Top, center) Eto^- or Ctr^- mutants that are identified as mutants that display the seedling triple response grown on agar media in the dark without the addition of exogenous ethylene or its precursors. (Bottom, center) Ein^- mutants are identified because they fully lack the ability to respond to ethylene when grown in the dark in the presence of ethylene. Tissue specific mutants are selected by identifying the organs that do not respond to ethylene treatment (not shown).

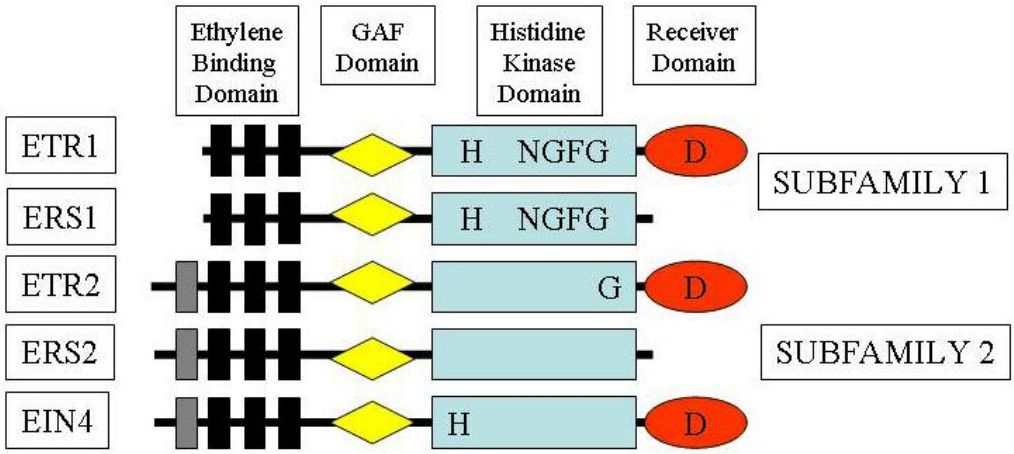
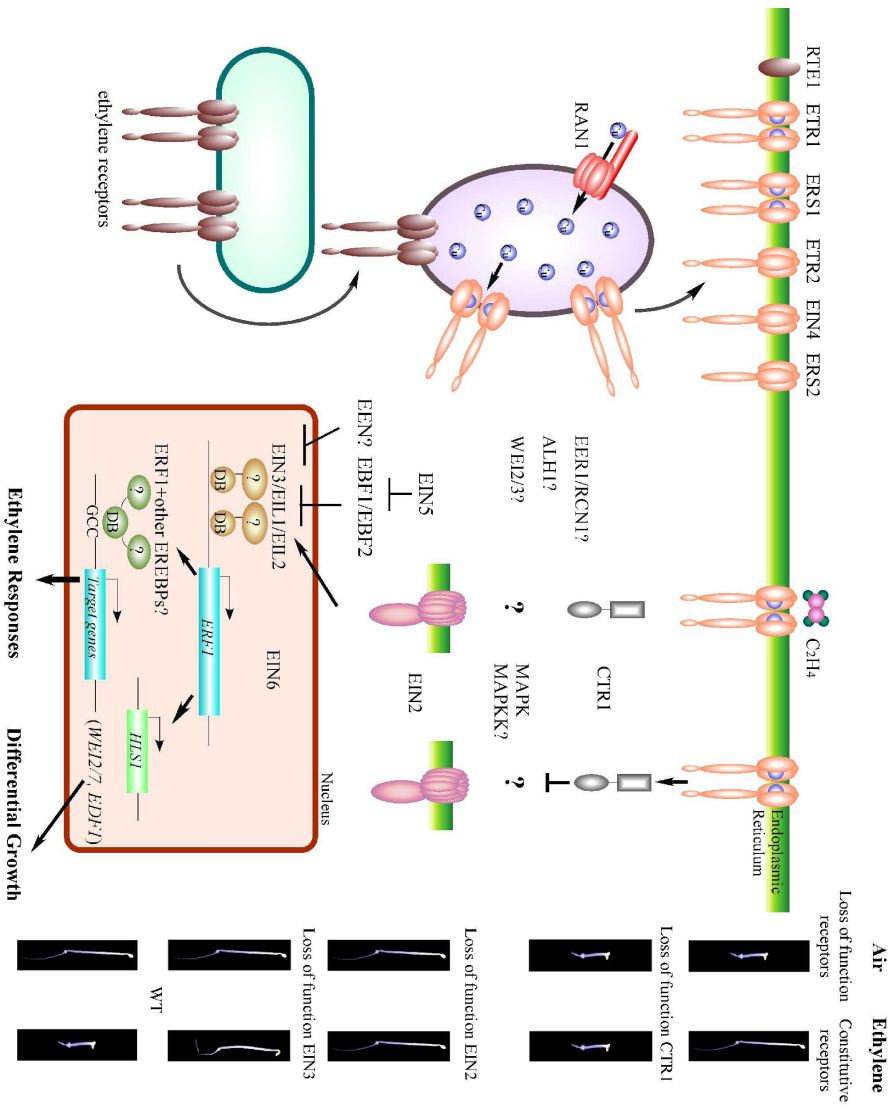


Figure 1.2. The structure of the ethylene receptor family of *Arabidopsis thaliana*.

The ethylene binding domain is located in the transmembrane section of the protein. The GAF domain of unknown function lies next to ethylene binding domain. All receptors contain a histidine kinase domain. The presence of the receiver domain is variable. Histidine (H) and Aspartate (D) phosphorylation sites are indicated. Only ETR1 and ERS1 contain conserved sequences in the histidine kinase domain required for activity (H, N, G, F, G), this allows the receptors to be divided into two subfamilies based upon sequence and phylogenetic analysis.

Figure 1.3. A model for the ethylene signal transduction pathway

Five transmembrane proteins, ETR1, ETR2, ERS1, ERS2, and EIN4, make up the family of ethylene receptors in *Arabidopsis*. Ethylene binding occurs at the N-terminal transmembrane domain of the receptors, and a copper co-factor is required for the binding. RAN1, a copper transporter, is involved in the delivery of copper to the ethylene receptor. In the absence of an ethylene signal, ethylene receptors activate a Raf-like kinase, CTR1, and CTR1 in turn negatively regulates the downstream ethylene response pathway, possibly through the MAP-kinase cascade including SIMKK, MAPK6 and MAPK13. Binding of ethylene inactivates the receptors, resulting in the deactivation of CTR1, which allows EIN2 to function as a positive regulator of the ethylene pathway. EIN2 contains an N-terminal hydrophobic domain similar to the Nramp metal transporter proteins and a novel hydrophilic C terminus. EIN2 positively signals downstream to EIN3 and its family of related transcription factors, located in the nucleus. EIN3 binds to the promoter of ERF1 and activates its transcription in and ethylene-dependent manner. The transcription factors ERF1 and the other EREBPs can interact with the GCC box in the promoter of target genes to activate the downstream effects of the ethylene pathway.


Air
Ethylene
Loss of function receptors
Constitutive receptors
Endoplasmic Reticulum
C2H4
RTE1 ETR1 ERS1 ETR2 EIN4 ERS2
RAN1
ethylene receptors
EER1/RCN1?
ALH1?
WEI2/3?
CTR1
MAPK
MAPKK?
?
?
Loss of function CTR1
EIN5
EIN2
Loss of function EIN2
EEN? EBF1/EBF2
EIN3/EIL1/EIL2
EIN6
DB
ERF1
ERF1+other EREBPs?
GCC
target genes
HLS1
(WEI2/7, EDF1)
Nucleus
WT
Loss of function EIN3
Ethylene Responses
Differential Growth

CHAPTER II

Cloning and Characterization of *ETHYLENE INSENSITIVE SIX* and the *ENHANCER OF ETHYLENE INSENSITIVITY*

ABSTRACT

Ethylene is a gaseous plant hormone that affects diverse processes throughout plant growth and development. Its biosynthesis is mediated by the plants responses to both biotic and abiotic stresses. Nearly two decades of work in *Arabidopsis* has evolved into ethylene having one of the best characterized signal transduction pathways of all plant hormones. Here we describe two new ethylene mutants that act synergistically to cause an ethylene insensitive seedling phenotype. *ETHYLENE INSENSITIVE6* (*EIN6*) when mutated causes an ethylene insensitive root phenotype in *Arabidopsis* etiolated seedlings. The protein encoded by this gene shares homology with jumonji domain proteins that have recently been implicated in histone demethylase activity in other eukaryotic organisms. Additionally *ein6* mutants display a pleiotropic range of microtubule associated phenotypes not previously documented for other ethylene mutants. Mutants in the gene *ENHANCER OF ETHYLENE INSENSITIVITY* (*EEN*) do not demonstrate an obvious phenotype on their own, but enhance the ethylene insensitive root phenotype of *ein6* plants to a full ethylene insensitive etiolated seedling phenotype. This is the first enhancer mutation found in the ethylene signal transduction pathway. This study reveals a new component of the ethylene signal transduction pathway that may be associated with epigenetic control of transcriptional regulation by modification of core histones.

INTRODUCTION

The hormone ethylene is an olefin hydrocarbon produced by all plants. Despite its simple chemical structure it orchestrates a myriad of complex functions. Ethylene controls processes as diverse as germination, root hair development, root nodulation, senescence of organs (including fruit ripening), differential cell growth, abscission, stress responses and resistance to necrotrophic pathogens (Abeles et al., 1992; Abel et al., 1995) Acting as a hormone ethylene is known to be able to bring on biological responses at nanomolar concentrations and that its response only takes minutes to be induced. Due to the key importance of ethylene throughout the plant growth cycle, the production of this hormone is a tightly regulated process, controlled by both developmental signals and response to environmental stimuli.

Many of the components of the ethylene biosynthesis and signal transduction pathways have been isolated by powerful genetic screens in *Arabidopsis thaliana*. Exposure to ethylene induces dramatic morphological changes in the growth of seedlings. Normally an etiolated (dark-grown) seedling displays a closed apical hook, a long slender hypocotyl and an elongated root. When exposed to ethylene seedlings exhibit exaggerated curvature of the apical hook, radial swelling of the hypocotyl, and inhibition of hypocotyl and root elongation. This triple response phenotype has been employed to find mutants which do not correctly respond to exogenous ethylene treatment (Guzman and Ecker, 1990). Ethylene is produced from the amino acid methionine via the Yang Cycle (Yang and Hofmann, 1984) by a series of well characterized enzymatic reactions. The rate limiting step is the conversion of S-

andenosyl-Methionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) by the ACC synthetase (ACS) enzyme family (Wang et al., 2002). These genes have a differential expression pattern through tissues of the developing and adult plant and different isoenzyme diversity among cells and tissues within the plant (Tsuchisaka and Theologis, 2004, 2004). ACS activity is regulated at both the transcriptional and posttranscriptional levels (Chae and Kieber, 2005). Following biosynthesis ethylene is recognized by a family of five ER membrane-localized receptors (Chen et al., 2002). These receptors have homology to bacterial two-component histidine kinase receptors. The receptors bind ethylene in copper dependant manner and require the copper transporter, *RESPONSE TO ANTAGONIST* (*RAN1*), for ligand specificity (Hirayama et al., 1999). The receptors physically interact with the next member of the pathway, *CONSTITUTIVE TRIPLE RESPONSE1* (*CTR1*), which acts as a negative regulator of the ethylene pathway. In the absence of ethylene, the receptors are active and stimulate the kinase activity of CTR1, allowing it to possibly phosphorylate downstream targets and causing a repression of downstream signaling (Huang et al., 2003). *ETHYLENE INSENSITIVE2* (*EIN2*), acts as a positive regulator as the next step in the ethylene signaling cascade. EIN2 encodes a large membrane bound protein which acts in an unknown manner to activate the transcription factor EIN3 and a family of EIN3-LIKE (EIL) proteins. EIN3 protein levels are regulated in a post transcriptional manner by two F-box proteins, EIN3 BINDING FACTOR1 and 2, (EBF1 and EBF2) via degradation through the ubiquitin/26S proteosome (Guo and Ecker, 2003; Potuschak et al., 2003). The transcripts of *EBF1* and *EBF2* were

recently found to be controlled by *EIN5/XRN4*, a 5' to 3' exoribonuclease, and therefore the turnover of EIN3 (Olmedo et al., 2006; Potuschak et al., 2006). EIN3 and the EILs act as transcriptional regulators of ethylene responses by binding to the promoters of *ETHYLENE-RESPONSE ELEMENT BINDING PROTEINS* (*EREBP*s), this binding serves to activate these EREBPs and other transcription factors and stimulates ethylene responses at the molecular level.

The mutant, *ein6*, was identified in a previously conducted genetic screen for Arabidopsis seedlings that have an aberrant triple response phenotype, specifically to have a recessive insensitivity to ethylene (Roman et al., 1995). Unlike *etr1* and *ein2*, *ein6* mutants were found to retain some sensitivity to ethylene, with the root being more sensitive then the hypocotyl. This mutant was thought to have reduced gameophytic transmission and therefore have a reduced number of Ein^- progeny resulting from a backcross to wild-type seedlings. Further analysis of this mutant revealed that two mutant loci are interacting to cause the ethylene insensitive phenotype, not reduced gametophytic transmission, the first locus *is ETHYLENE INSENSTITIVE6* and the second is the *ENHANCER OF ETHYLENE INSENSITIVITY* (*EEN*). This paper describes the isolation and characterization of *EIN6*, along with mapping of its enhancer *EEN*, two genes required for normal ethylene responses.

RESULTS

Two Loci Cause the Ethylene-Insensitive Seedling Phenotype of ein6

The *ein6* mutant was previously identified from a genetic screen for classical triple-response mutants (Roman et al., 1995) (Figure 1A). It was found to have an increased resistance to exogenous ethylene treatment. This mutant was not allelic to any previously described ethylene mutants. The mutant retained some sensitivity to ethylene with a less severe growth response then other well characterized ethylene insensitive mutants such as *ein2* or the *ein3eil1* double mutant (Figure 1B). These plants were also thought to have a reduction in gametophytic transmission, based upon the number of Ein^- progeny resulting from a backcross to wild type plants. Upon further examination of a backcross population it was discovered that there are three resulting phenotypes when F2 seeds were germinated on ACC plates in the dark. These phenotypes were Ein^+ (wild type), Ein^- and an intermediate ethylene-insensitive root phenotype (Eir^-). These phenotypes were found to significantly fit the Mendelian inheritance pattern of 12:3:1 (Ein^+, Eir^-, Ein^-) (data not shown), indicating the presence of two mutant loci in the original mutant background. When the *ein6* locus alone is mutant the resulting phenotype is an ethylene insensitive root (Figure 1B). When the *ein6* locus is mutant and the *enhancer of ethylene insensitivity* (*een*) is also mutant this results in a fully ethylene insensitive seedling (Figure 1A and 1B). A plant containing only the *een* mutation does not display any known phenotype that is distinguishable from wild-type seedlings. A comparison of the seedling phenotypes to other ethylene insensitive mutants, when grown in the presence of ACC, can be seen in figure 1B.

Adult ein6een plants have a mild leaf phenotype, with a more round leave morphology, characterized by shorter petioles and leaf blades. This leaf morphology may be indicative of a greater number of juvenile leaves versus wild-type plants during the vegetative stages of development (Telfer et al., 1997).

ein6 seedlings alone display an ethylene insensitive root phenotype similar to that seen in a mutant from the same screen by Roman et al, eir1. Upon cloning the *EIR1* locus it was found to be an auxin efflux carrier (Luschnig et al., 1998). This phenotype has often been correlated to a disruption in polar auxin transport and has been noted for a variety of auxin mutants such as *axr1*, *axr2* and *aux1*. *ein6* seedlings show insensitivity to the ethylene precursor ACC in the root, but a normal wild-type response to ACC in the hypocotyl.

ein6 and een are Epistatic to ctr1 in Seedlings and Adult Plants

As previously documented ein6 is epistatic to the constitutive ethylene signaling mutant *ctr1* (Roman et al., 1995). Both *ein6* and *een* are epistatic to *ctr1* and when the triple mutant cross is constructed from these mutants, they are able to rescue the constitutive signaling phenotype of *ctr1* plants back to a wild type response to ethylene. Plants mutant for *ctr1* have a severe phenotype in both seedling and adult phases of their life cycle. As etiolated seedlings *ctr1* displays a dramatic triple response phenotype when grown in air, mimicking the phenotype shown by wild-type seedlings when grown in the presence of exogenous ethylene. As adults *ctr1* plants are extremely dwarfed in stature with a severe reduction in the size of both the inflorescence and the rosette leaves (Kieber et al., 1993). The rescued phenotype of

ctr1 plants is visible in both etiolated seedling and adult phases of the plants life cycle (Figure 2 A and B).

The interaction played out between *ctr1* and these mutants can also be visualized at the molecular level via plants expressing transcriptional reporter fusions between the promoter of CTR1 and β-*GLUCURONIDASE* (GUS). *CTR1-GUS* is a strong ethylene inducible promoter fusion, which displays significant induction of *CTR1* transcript in the hook region of seedlings treated with exogenous ethylene (Figure 2 C and D). When this reporter is introduced into *ein6een* seedlings and the level of induction caused by ethylene treatment was greatly reduced suggesting a lower level expression of the *CTR1* gene in these mutant plants (Figure 2 E and F).

Cloning the EIN6 Locus

ein6 had been mapped to the short arm of chromosome 3, north of the marker nga112 (Roman et al., 1995). Further mapping using SSLP (Simple Sequence Length Polymorphism) markers (Bell and Ecker, 1994) and a mapping population of 500 F2 plants generated by a cross between the accession Niederzenz (Nd-0) and *ein6een* plants was used to narrow the mapping region to a 12 cM interval between the markers TOPP5 and g19397 on chromosome 3 (Figure 3A). Further mapping using markers developed from cloned genes in the region and BAC end sequences located the mutation to the BAC T29H11. Final mapping narrowed the region a 44kb interval of genomic DNA (see methods). Although the *ein6* mutation was induced by fast neutron mutagenesis, which normally causes large chromosomal aberrations, there was no evidence of a deletion present in the region when genomic southern blots of

the mutant were probed with the BAC F3E7, which overlapped the mapping region (data not shown). Because genomic sequence for the region was available Denaturing High Performance Liquid Chromatography (DHPLC) was chosen as a method capable of identifying single base mismatches in double stranded DNA (O'Donovan et al., 1998). Primers were designed to amplify overlapping ~500bp PCR fragments that completely covered the 44kb interval between the two closest recombinant markers. Elution from the DHPLC column yields a single peak if no mismatch is present or multiple peaks if a mismatch is present. Multiple peaks were only obtained from one PCR fragment within the 44kb region. Sequencing of the fragment from wild-type and mutant plants revealed a 7 bp deletion in the mutant fragment.

This 7 bp deletion was present in the gene At3g48430, previously found to be related to an early flowering mutant, and called *RELATIVE OF EARLY FLOWERING6* (*REF6*) (Noh et al., 2004). The predicted 1360 amino acid protein is encoded by a 4083 bp cDNA (Figure 3B and C). The mutation was found to be located in the fifth exon of the predicted protein. The deletion causes a missense mutation, at amino acid 167, and a frameshift which results in a premature stop codon after amino acid 181. Using the genomic DNA for the predicted At3g48430 gene and 2.2 kb of its promoter the ethylene insensitive double mutant phenotype can be rescued to a wild-type phenotype (Figure 3D). There are a number of conserved domains within this predicted protein, including both a Jumonji N and a Jumonji C domain and three C2H2 zinc fingers. Proteins containing the jumonji C domain were thought to act as transcriptional regulators but have recently been shown to function as

a histone demethylases (Klose et al., 2006). EIN6 contains the conserved amino acids that are required for enzymatic activity (Figure 3E), as shown by an alignment to two different jumonji histone demethylases from humans.

EIN6 Expression is not Ethylene Inducible

EIN6 is expressed at extremely low levels, low enough that its expression cannot be visualized by a northern blot (data not shown). There are no probes contained within the *EIN6* gene present on the Affymetrix ATH1 expression array, so expression can not be analyzed utilizing available expression array data either. Expression is visible by RT-PCR and has been seen in all tissues tested (data not shown). To further investigate the expression of the *EIN6* gene a fusion between 2.5 kb of the *EIN6* promoter (2.5 kb upstream of the predicted start codon) and the reporter gene GUS were constructed. This fusion was transformed into wild-type seedlings along with both *ein6-1* and *ein6een* double mutant seedlings. Seedlings were stained after 3 days grown in the dark either in the presence or absence of 10μM ACC. All seedlings showed diffuse staining that did not appear to accumulate in an ethylene dependant manner (Figure 4). Seedlings grown in the presence of ACC showed the same level of staining as those grown without exogenous ethylene treatment. Staining was concentrated in the vasculature, particularly that of the cotyledons, at the root and hypocotyl junction, and the shoot apical meristem and leaf primordia. The staining pattern did not change in the presence of the *ein6-1* or *een* mutations (data not shown).

Non-Ethylene Insensitivity Related Phenotypes of ein6

Although *ein6-1* and *een* were found in a screen for ethylene insensitive mutants they have a number of phenotypes not usually associated with ethylene insensitivity. These ethylene insensitivity-independent phenotypes can all be related to microtubules. There are known relationships between ethylene and microtubule organization. The role of microtubules in cell elongation has long been thought to be a downstream effect of ethylene treatment due to the fact that application of exogenous ethylene causes reorientation of cortical microtubules from a transverse to longitudinal direction in pea epicotyl cells (Apelbaum and Burg, 1971). The change in microtubule direction then causes an inhibition of cell expansion, which may be read out as the shortened hypocotyl and root of the "triple response" (Shibaoka, 1994). This reorientation in the *Arabidopsis* root epidermis is rapid, starting within 10 minutes of ACC treatment and is completed within an hour (Le et al., 2004). The same study showed ethylene induced reorientation to be position and cell type dependent. In a previous study *ein6een* mutants have been shown to have a hypersensitivity to the drug taxol, which acts to stabilize microtubules against depolymerization (Bokros et al., 1993; Roman and Ecker, 1995). At a concentration 10 fold less then that which affects wild-type seedlings (0.1μM versus 1μM) e*in6een* double mutant seedlings, but not *ein6-1* seedlings show severe cell swelling and overall alterations in their cell architecture. Other ethylene dependent mutants did not display this hypersensitivity to taxol (Roman and Ecker, 1995). Figure 5A shows a comparison between wild-type (Ler) seedlings, *ein6-1* and *ein6een* seedlings all grown for 10 days in the dark with and without 0.1μM taxol. Wild-type seedlings appear to

have little difference between the treatments, whereas *ein6-1* shows some effects of low-concentration taxol treatment (slightly shorter) and *ein6een* is acutely effected.

An additional mutant defect of the *ein6een* mutant combination is in the root gravitropic response. When Arabidopsis is grown on vertical hard agar surfaces roots grow in a wavy pattern in response to the root tip not being able to penetrate the agar surface when gravitropism directs its downward growth (Okada and Shimura, 1990). The parental ecotype of the *ein6een* mutant, Ler, is known to have a mild right handed skewing of its roots when compared to other ecotypes such as Col (Figure 5 B Col-0 vs. Ler-0 panel). The right handed growth of Ler can be corrected by treatment with exogenous ethylene or by growing plates under conditions that do not allow for gas exchange and cause an accumulation of ethylene (Buer et al., 2003). Additionally this same study showed that ethylene causes an increase in the tightness of root bending angles. Although not seen in other ethylene mutants, the auxin hormone mutants, *aux1* and *eir1*, have been found to be agravitropic in the root waving response, demonstrating that roots require the activity of an auxin influx and efflux carrier for gravitropic response (Luschnig et al., 1998; Marchant et al., 1999). *AUX1* has further been shown to be essential for regulating differential expansion of root epidermal cells which causes the gravitropic root bending response (Swarup et al., 2005). *ein6een* roots display an agravitropic phenotype and exaggerated right handed skewing in comparison to wild-type Ler or other ethylene insensitive mutants (Figure 5B right panel). The roots of the mutant do not have any regular waving pattern and actually form coils periodically instead of growing down the surface of the agar showing an

enhanced directional growth bias. Although the auxin gradient has been shown to be essential for root bending, so have cytoskeletal related genes such as α tubulins in the establishment of cell polarity associated with gravitropic growth (Bao et al., 2001; Thitamadee et al., 2002). Regulation of root tip rotation is thought to be controlled by the arrangement of cortical microtubule arrays (Hashimoto, 2002). Perhaps linking the microtubule defect seen by hypersensitivity to taxol in *ein6een* to its root phenotype.

Wild-type, Ler, plants display three branched trichomes with nearly tetrahedral geometry on rosette leaves (Perazza et al., 1999) (Figure 5C left panel). Three branched trichomes make up over 80% of the trichomes present on wild-type leaves. *ein6-1* and *ein6een* plants have a trichome branching defect, where both mutant combinations display trichome overbranching (Figure 5C middle and right panel). This is not a phenotype that has been noted for other ethylene insensitive mutants, as a result trichomes from *ein2-5* and *ein3-1* were examined and noted to be wild-type in branch number (data not shown). Some supernumerary trichome branching mutants result from an increase in trichome cell size or higher DNA content of trichomes caused by an increase in endoreduplication cycles (Schwab et al., 2000). The cytoskeleton also plays a key role in trichome branching, with tubulin being involved in trichome branching and actin being involved in guiding the direction of growth (Mathur et al., 1999).

Map Location of the EEN Locus

Mapping of the *EEN* locus was not concurrent with mapping of the *EIN6* locus. Using a new mapping population of *ein6een* plants crossed to Col-0, initial mapping analysis revealed two areas of linkage in this population using 26 SSLP markers spread along the 5 chromosomes of *Arabidopsis* (Bell and Ecker, 1994). The first area of linkage corresponded to the location of the *EIN6* locus on the short arm of chromosome 3. The second area of linkage corresponded to the markers NGA 1139 and NGA 1107, located near the end of chromosome 4. Additional SSLP markers were designed from repeat sequence from Ler and Col, and narrowed the location of the mutation to a 73 gene region on BACs F20D10 and F22I13 (Figure 6). There are no known ethylene genes in this region, although the HLS1 locus fell in the larger mapping region on BAC F19F18, supporting the fact that *een* is a novel locus in ethylene signaling, rather then an allele of a previously characterized ethylene mutant.

DISCUSSION

In this report we show both ethylene dependent and independent phenotypes of two previously uncharacterized *Arabidopsis* ethylene signaling mutants. The first novel aspect of this study is the nature of the mutants, being discovered originally as a double mutant and turning out to be a mildly insensitive mutant combined with an enhancer to cause the fully ethylene insensitive phenotype. Additionally these mutants seem to be both important for proper transduction of the ethylene signaling cascade and also important for microtubule organization. These two phenotypic classes of mutants have not been previously associated with each other, but could intersect as a downstream effect of ethylene on microtubule re-orientation.

Role of EIN6 and EEN in the Ethylene Response

A combination of two mutations in *ein6* and *een* result in an ethylene insensitive seedling. This is the first enhancer mutation identified to be involved in the ethylene signaling cascade. It was a fortuitous discovery that came from two simultaneous mutations that were generated during the fast-neutron irradiation mutagenesis. This is not the only case of simultaneous mutations caused by fast-neutron irradiation, Ohshima and colleagues characterized a recessive mutation that was generated concurrently with a single dominant modifier during mutagenesis (Ohshima et al., 1998). In addition to phenotypic evidence that *ein6* and *een* are required for functional ethylene signal transduction they have also been shown to be essential for EIN3 protein accumulation (Guo and Ecker, 2003). EIN3 protein accumulates in an ethylene dependent manner, with increased protein levels noted

after an hour of exogenous ethylene treatment. This protein accumulation is dependent on an intact ethylene signaling cascade; ethylene insensitive signaling mutants cause a dramatic decrease (*ein4, etr1, ein5*) or abolishment (*ein2*) of protein accumulation. In the double mutant, *ein6een*, there is no EIN3 protein accumulation, similar to what is seen for the *ein2* mutant (Guo and Ecker, 2003). This decrease in EIN3 protein accumulation is dependent *EEN*, because in the *ein6-1* mutant EIN3 protein accumulates to normal levels (Guo and Ecker, personal communication). The regulation of the EIN3 protein by *EIN6* and *EEN* appears to be at the post-translational level, since transcript levels of *EIN3* are unchanged in either *ein6een* or *ein6-1* (Nehring and Ecker, unpublished). It is interesting that *een* does not have an ethylene related phenotype on its own as mutants in other genes that control the post-transcriptional modification of EIN3, such as *EBF1* and *EBF2*, have a *ctr1*-like phenotype.

EIN6 Acting as a Transcriptional Activator?

Until recently it was unclear if the epigenetic mark of histone methylation was reversible. Now three classes of enzymes have been found to modify histone methylation or act as demethylases (Klose et al., 2006). The largest class of these is the Jumonji C (Jmj C) domain containing proteins. New studies in the last year have shown Jmj C proteins can act as histone demethylase enzymes. Jumonji C domain containing histone demethylases (JHDMs) were initially isolated biochemically from cell fractionations that were shown be active in catalyzing lysine demethylation and required α-ketoglutarate and iron as cofactors in the reaction (Klose et al., 2006). The

Jmj C enzymes have now been shown to be specific for demethylating different histone lysine marks and different methylation states, H3K36, H3K9, H3K36 and H3K9 (Klose et al., 2006) or most recently H3K4 (Klose et al., 2007). Different histone lysine marks represent different transcriptional states, where H3K27, H3K9 and H4K20 are usually marks of heterochromatic or silenced DNA and H3K4, H3K36 and H3K79 are usually marks of euchromatic or actively transcribed DNA (Martin and Zhang, 2005).

Thirty Jmj C proteins have been identified in humans alone, these fall into 7 phylogenetic categories (Klose et al., 2006) not all of which have been shown to have histone demethylase activity. There has been no histone demethylase published in plants to this date. EIN6 shares homology with these Jmj C domain containing proteins. Specifically it appears to be most closely related to the JARID1 family of histone demethylases. This JARID family, including member RBP2 or JARID1a, was recently characterized as removing the histone methylation mark from tri-methylated histone H3 lysine K4 *in vivo* (Klose et al., 2007). This is a mark of actively transcribed DNA. There are a number of key residues required for α-ketoglutarate and Fe(II) binding that appear to be conserved throughout family members, the EIN6 protein contains all of these residues which makes it a promising candidate as a plant H3K4 demethylase (see Figure 3D for a comparison of EIN6 to RBP2 and JHDM3A). Currently there is no full length cDNA cloned for EIN6, which would be useful in determining *in vitro* and *in vivo* function as a histone demethylase through assays developed by other groups. Until the cDNA is generated other methods could be

employed for association of EIN6 as a demethylase. These methods include chromatin immunoprecipitation (ChIP) coupled to Arabidopsis whole-genome array (WGA) analysis with an anti-tri methyl H3K4 chip grade antibody which would identify EIN6 protein interacting factors, including target DNA fragments on a whole genome level. Additionally western blots to assay for overall change in histone H3K4 level and yeast two-hybrid screening to identify EIN6 interacting proteins would also be useful in linking EIN6 to histone demethylation. As the story of the Jmj C domain unfolds it will be interesting to see if EIN6 plays a role in the plant kingdom.

Ethylene independent roles of EIN6 and EEN

In addition to the ethylene related phenotypes seen in *ein6een* plants they also have a number of ethylene independent phenotypes that may be associated with changes in microtubule organization. These phenotypes are hypersensitivity to the microtubule stabilizing drug taxol, gravitropism defects and trichome overbranching. Although it is fairly common to see two of the three of these phenotypes correlated to each other, there are no cases where all three of these phenotypes have been noted before.

Taxol functions by stabilizing microtubules against depolymerization by binding to the microtubules (Bokros et al., 1993). Hypersensitive mutants have mainly been discovered to be members of tubulin protein families (Gaertig et al., 1994; Thitamadee et al., 2002) or as proteins of unknown function (Sedbrook et al., 2004). It was found that treatment of some right hand skewed gravitropic mutants with low levels of taxol can reverse root skewing (Furutani et al., 2000), this was not

tested on the *ein6een* mutant. This effect of microtubule interacting drugs on agravitropic root growth is thought to be caused by a change in microtubule growth or orientation.

Agravitropic root growth is thought to be caused by either abnormal auxin transport or change in the helical alignment of microtubules in epidermal cells of the root elongation zone. When roots exhibit skewing right handed skewing is associated with left handed helical alignment of microtubules and left handed skewing is associated with right handed helical alignment of microtubules (Furutani et al., 2000; Thitamadee et al., 2002). Transverse alignment of both microtubules and cellulose microfibrils is required in the elongation zone to have proper gravitropic root growth. It has been determined that cortical microtubules are not directly involved in the signal for the direction of cellulose microfibril deposition (Sugimoto et al., 2003). Although if either of these components of the cell architecture is disrupted it results in plants agravitropic root growth.

Trichome branching can be changed by transiently stabilizing microtubules via drug treatment with taxol. This can result in a change in branch position and length, new branch points and an overall increase in trichome branching (Mathur and Chua, 2000). Trichome branching and polarized growth requires the synthesis of new microtubules (Szymanski et al., 2000). Trichome branching mutants fall into one of two categories the first having an increase in DNA content of the cells where the more copies of DNA equals more trichome branches (Schwab et al., 2000) and the second which does not have an increase in DNA content (Folkers et al., 1997).

There is one characterized mutant associated with ethylene signaling which has nearly a converse phenotype of *ein6een*. This mutant, *polaris*, *pls*, has an enhanced ethylene signaling seedling phenotype when air grown in the dark, this less severe then *eto1* or *ctr1* (Chilley et al., 2006). *pls* also displays less sensitivity to microtubule inhibitory drugs such as oryzalin, which causes similar radial cell swelling phenotypes as taxol (Baskin et al., 1994). This phenotype lead to the prediction that the *pls* mutants are defective in stabilization of microtubules, which would cause radial cell swelling and the enhanced ethylene response and short root phenotypes of *pls* (Chilley et al., 2006). This report taken with the phenotypes of *ein6* and *ein6een* would lead to the conclusion that these two mutants cause a destabilization of microtubules, which leads to the hypersensitivity to taxol, root agravitropism caused by incorrect orientation of cortical microtubule helical arrays and possibly the change in trichome branching.

There are a diverse set of phenotypes associated with the mutant combination of *ein6* and *een*. The genes are involved in the proper transduction of the ethylene signal through the transcription factor EIN3, they also play a role in microtubule stability and the EIN6 might function as a regulator of transcription by removing methylation modification from core histones. Additionally since the exact nature of the *een* mutation is yet to be determined identification of the gene responsible for this mutation will be key in understanding how these two mutants work synergistically to cause this range of phenotypes. If EIN6 acts as a demethylase to remove the mark of active transcription, then in the mutant there should be more areas of active

euchromatin then in wild-type, its role in ethylene signaling could be explained if its targets are genes which control the stability of EIN3, such as *EBF1* or *EBF1*. Determining whether it functions as a histone demethylase and identifying the targets of EIN6 by chIP chip will shed light on how this gene controls a wide range of processes.

MATERIALS AND METHODS

Arabidopsis Strains and Growth Conditions

The *ein6een* mutant was generated in the Ler background as previously described as *ein6* by Roman et al., 1995. The *ctr1* allele used for epistasis crosses was also in the Ler background and was generated in the Ecker lab. Other ethylene mutant seeds used for phenotypic comparison, *etr1-1*, *ein2-1*, *ein2-5*, *ein3eil1*, *ein3-1*, *ein5-1*and *eir1-1* were all in the Col-0 background. The *CTR1-GUS* reporter line (*T116-GUS*) was previously constructed in the Ecker lab and was a generously provided by Hai Li.

For planting on plates seeds were sterilized either by vapor phase sterilization (Clough and Bent, 1998) or surface sterilization. Surface sterilization was carried out in a microcentrifuge tube containing less then 150ul of *Arabidopsis* seed. Seeds were first rinsed with 70% ethanol to decrease surface tension and then washed for 10 to 20 minutes with a solution of 50% commercial bleach (2.625% sodium hypochlorite, final volume) containing 0.04% Triton X-100 (Sigma-Aldrich). Seeds were then rinsed three times with sterile distilled water. Seeds were plated using a 0.1% agarose solution prepared in sterile distilled water. Seeds were plated onto agar plates containing 1 X Linsmaier and Skoog salts buffered to pH 5.7 (Caisson Labs Inc.), with 8g/L sucrose and 1.2 to 1.8% agar (Caisson Labs Inc.), unless otherwise noted. Plates were supplemented with 10μM ACC or 0.1μM taxol when noted. Seeds were stratified for 3 days at 4 degrees Celsius in the light after being sowed on plates then exposed to light for 2 hours prior to being wrapped in aluminum foil for growth in the

dark. Etiolated seedlings were grown for 3 days in the dark at 24 degrees Celsius, unless otherwise noted. For propagation dark grown seedlings were transferred to soil and sowed in prewetted Pro-Mix HP (Premier Horticulture) mixed 3:1 with Vermiculite (medium to coarse grade) and treated with Adept (prepared at a concentration of 1 oz per 4L of water) (Uniroyal Chemicals) and Marathon 1% granular insecticide (Olympic Horticultural). Plants were grown at 24 degrees Celsius in long day growth conditions cycling 16 hours of light and 8 hours of dark.

Mapping and Cloning ein6

Initial mapping of the *EIN6* locus was performed using F2 progeny of a cross between *ein6een* and wild type plants of the Columbia (Col-0) ecotype. Markers used for mapping were Simple Sequence Length Polymorphisms (SSLP (Bell and Ecker, 1994)) or Cleaved Amplified Polymorphic Sequences (CAPS (Konieczny and Ausubel, 1993)). Subsequent fine mapping was performed using F2 progeny of a cross between *ein6een* and wild type plants of the Niederzenz (Nd-0) ecotype (provided by Saeid Nourizadeh, University of Pennsylvania). Mapping was performed using a population of 496 F2 plants, and data were quantified and linkage assessed using Map Manager QT software (http://mcbio.med.buffalo.edu/mapmgr.html). Markers used for mapping include the following primers found in table 2.1.

Table 2.1: Primers for mapping the *ein6* locus

Marker	Primers	Type	Enzyme	Reference
GL1a	5'ATATTGAGTACTGCCTTTAG3' 5'CCATGATCCGAAGAGACTAT3'	CAPS	Taq I	Konieczny and Ausubel, 1993
nga112	5'TAATCACGTGTATGCAGCTGC3' 5'CTCTCCACCTCCTCCAGTACC3'	SSLP	n/a	Bell and Ecker, 1994
BGL1a	5'TCTTCTCGGTCTATTCTTCG3' 5'TTATCACCATAACGTCTCCC3'	CAPS	Rsa I	Konieczny and Ausubel, 1993
PUR5	5'GATGTAGACCTTGCTGAAAA3' 5'AAACCTTTCACTCCTCCTTTTTC3'	CAPS	Rsa I	unpublished
g19397	5'CCGACAGTGGAATGCAGAGTTC3' 5'AGATGTAAGCAAGGCAAGCACC3'	CAPS	Hae III	K. Schrick, personal communication
TOPP5a	5'TCGACGACATCATTCGTCGT3' 5'GAACTGAAGCATCCTGCAGT3'	CAPS	Rsa I	unpublished
EDS1	5'AGGAACTGGTACAGTCGATG3' 5'CAGCTTGAACGTACTGTCTG3'	SSLP	n/a	unpublished
F1K9-SP6	5'GCAATCTTCATCTCCCTAAACG3' 5'GCTTGCAAGCTCAACTAACC3'	CAPS	Hinf I	unpublished
F1K9-T7	5'CCGAGACTTCCTCTGATAAG3' 5'ATCCATGCGAGCCACGATAC3'	CAPS	Hpa II	unpublished
29H11-End	5'CTGCGTTGTTTCAAGAAGTCC3' 5'GCTGAAGCACGTGCAACTAT3'	CAPS	Rsa I	unpublished

Table 2.1 continued

Marker	Primers	Type	Enzyme	Reference
F11N9	5'GCGATGCTTTTCTAAATCAGG3' 5'CGATATGGGGTTTATTTTGATCG3'	CAPS	Bsl I	unpublished
T11D3-SP6	5'CTTTCACTGCTTCAGTTAAAGC3' 5'GCGGTTGTCTCAGACAGGATAC3'	CAPS	ScrFI	unpublished
3E79	5'CCCTATAGACGCAAACACCAA3' 5'GTTTTGCTCTATAGTGATCTCG3'	CAPS	Rsa I	unpublished
15.1	5'CCTTTACTCTTGTTGCGTGC3' 5'AATGGCGAAGCAGCACTCGC3'	CAPS	Bsp HI	unpublished
12.1	5'GATATTTGCGAGACGCCACG3' 5'GCGTCATCGATGAACTGGAG3'	CAPS	Hpa II	unpublished
29/Mid	5'TTGGTGATAAATTAAGTGAAAGC3' 5'AGAAATCTGTTTGGTTCAATTG3'	CAPS	Bsp HI	unpublished
9.2	5'TCTCCATGTGAAGAGAAGCG3' 5'GCTCTGGGGATGTTGAAGTG3'	CAPS	Alu I	unpublished
7.3	5'ATGGTCACTTCGAGTAGCTC3' 5'GCGGGGTAACTTTACATGTC3'	CAPS	Dde I	unpublished
29/10	5'GCGTGTCGGTAGTTTCATCG3' 5'TTGCTTGTCGTCGGGTTTTC3'	CAPS	Dpn II	unpublished

Oligonucleotides for PCR were produced by either a Polyplex Oligonucleotide Synthesizer or Research Genetics (Huntsville, AL). Fragments were typically

amplified with an initial denaturation step of 45 s at 94° C, followed by 40 cycles of 94° C for 30 s, 55-60°C for 1 min, and 72° C for 1 min-2 min 30 s. PCR amplification was performed in a PTC-200 Thermal Cycler (MJ Research, Waltham, MA)

For DHPLC analysis, oligonucleotides based on the genomic sequence of BAC T29H11 (Accession #AL049659) were designed to amplify a series of overlapping PCR fragments of approximately 500 base pairs (bp) or less, that covered the genomic interval between the two closest markers that flanked the *EIN6* locus. Oligonucleotides were selected manually and assessed using Amplify software (http://www.wisc.edu/genetics/CATG/amplify/). The oligonucleotide sequences that were used to amplify a 456 bp fragment containing the *ein6* mutation were: forward 5'-GCAGCTGCTTCTTTAGGTTG-3' and reverse 5'-GCAGAGTGCCAACAAGCATC-3'. Individual fragments were amplified from wild type and mutant genomic DNA in 50 µl PCR reactions using cloned Pfu Polymerase (Stratagene, La Jolla, CA). In general, fragments were amplified using the Touchdown PCR method (Ault et al., 1994). Prior to DHPLC analysis, equal amounts of wild type and mutant fragments were mixed, denatured at 94° C in a PTC-200 Thermal Cycler, and allowed to cool to 25° C over 45 minutes. As controls, equivalent amounts of wild type and mutant fragments were separately denatured and reannealed under the same conditions. DHPLC was conducted on an automated HPLC instrument (The Wave; Transgenomic, Omaha, NE).

Sequencing of the mutant allele was done using BigDye Terminator version 3.1 kit (ABI Prism; PE-Applied Biosystems, Sunnyvale, CA).

Plant Transformation

The *EIN6-GUS* construct that contained a 2.5 kb promoter fragment generated by using PCR on genespecific primers fused into pB101.2 using XbaI and BamHI. Gene specific primers Promoter EIN6- F XbaI (5'-CCTCTCTAGAAATCATCTTCTTCTGTATTAGAAC-3') and Promoter EIN6- R BamHI (5'-CATTGGATCCATCTCTCTCTCTCTCTCACAC-3'). Then after cloning in the EIN6 promoter fragment upstream of GUS in pB101.2 the vector was sequenced using BigDye Terminator version 3.1 kit (ABI Prism; PE-Applied Biosystems, Sunnyvale, CA). The reporter was transformed into *Agrobacterium tumefaciens* and introduced into Ler, *ein6een* and *ein6-1* plants using the floral dip method (Clough and Bent, 1998). Primary transformants were selected on LS plates supplemented with 100 μg/mL kanamycin. Lines that segregated 3:1 for kanamycin resistance in T2 were propagated, and plants homozygous for the reporter were identified and used in GUS staining.

The EIN6-Genomic complementation clone was generated by digestion of the BAC T29H11 to yield a 6.2 kb fragment of genomic DNA encoding the EIN6 gene, this fragment was initially cloned into pBluescript II. This was subsequently directionally cloned into pROK1.2 using the restriction enzymes NotI and BamHI. This clone was transformed into *ein6een* and *ein6-1* plants as described above, with an empty pROK1.2 vector transformed into the same mutants as a control.

GUS Staining

Tissues were harvested and vacuum-infiltrated and stained for 72 hours in staining buffer [50 mM $NaP0_4$ buffer, pH 7.0, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$ and 1 mg/ml cyclohexylammonium salt]. Staining solution was rinsed four times with sterile liquid LS media, and individual representative seedlings were photographed.

Root Waving Assay

Root waving assays were performed essentially as originally described by Okada and Shimura (Okada and Shimura, 1990). Briefly, surface-sterilized seeds were stratified at 4° C for four days on MS medium (4.3 g/l MS salts (Gibco), 1% (w/v) sucrose, 1.5% (w/v) Bacto-agar, pH 6.0). Subsequently, plates were sealed with porous medical tape, and seedlings were grown vertically (90°) in 16 h:8H light: dark cycles at 24° C. After three days, plates were adjusted to 45° angles, and seedlings were allowed to grow for a further four days, at which point the root waving response was assessed. And representative seedlings were photographed.

EEN Mapping

A cross of ein6een to Col-0 plants was generated for mapping. Plants segregating the ethylene insensitive phenotype in the F2 were selected and tested for linkage using 26 SSLP markers (as described in EIN6 Mapping) across the 5 chromosomes of Arabidopsis. Subsequent fine mapping using a population of 500 phenotype selected F2 plants narrowed the region to the end of chromosome 4 using the following table of primers. Linkage was analyzed using web based linkage software, Sridhar Venkataraman and Natasha V. Raikhel, Arabidopsis Mapping Package using SSLP and CAPS data, 1999, http://www.msu.edu/~venkata1/sslpfull.htm

Table 2.2: Primers for mapping the *een* locus

Marker	Primers	Type	Enzyme	Reference
NGA1139	5'TTTTTCCTTGTGTTGCATTCC3' 5'TAGCCGGATGAGTTGGTACC3'	SSLP	n/a	Konieczny and Ausubel, 1993
NGA1107	5'CGACGAATCGACAGAATTAGG3' 5'GCGAAAAAACAAAAAAATCCA3'	SSLP	n/a	Bell and Ecker, 1994
HLS1	5'GTAATTATGCATTTATGCTATGG3' 5'GCATTCTAGGAAAAGTCCC3'	SSLP	n/a	Konieczny and Ausubel, 1993
T28I19	5'GGATTCTAACTACATTGGGA3' 5'GCTCATCTTGATTATATCT3'	SSLP	n/a	unpublished
F20D10	5'CATGTCCGTACATTATT3' 5'CCTATGACCATAATAAAG3'	SSLP	n/a	unpublished
F22I13	5'CCTGCATATATGTTAATTG3' 5'CGTTGCTTCAAAATATCCT3'	SSLP	n/a	unpublished
F20M13	5'GGATTCCTTGTTTTGCTTT3' 5'GCAGAATCGGAATCAAGA3'	SSLP	n/a	unpublished
T9A14	5'CCTTATGTTTGACAAAGAA3' 5'GGTTACTATACTGTTTTC3'	SSLP	n/a	unpublished
F19H22	5'GGCAAGGTAATTGATCGT3' 5'CCTCAATGAGTCCTTGAA3'	SSLP	n/a	unpublished
T22F8	5'GCTCCTAGGAATTCAATT3' 5'CGTCGTTTGAGGAGTTT3'	SSLP	n/a	unpublished

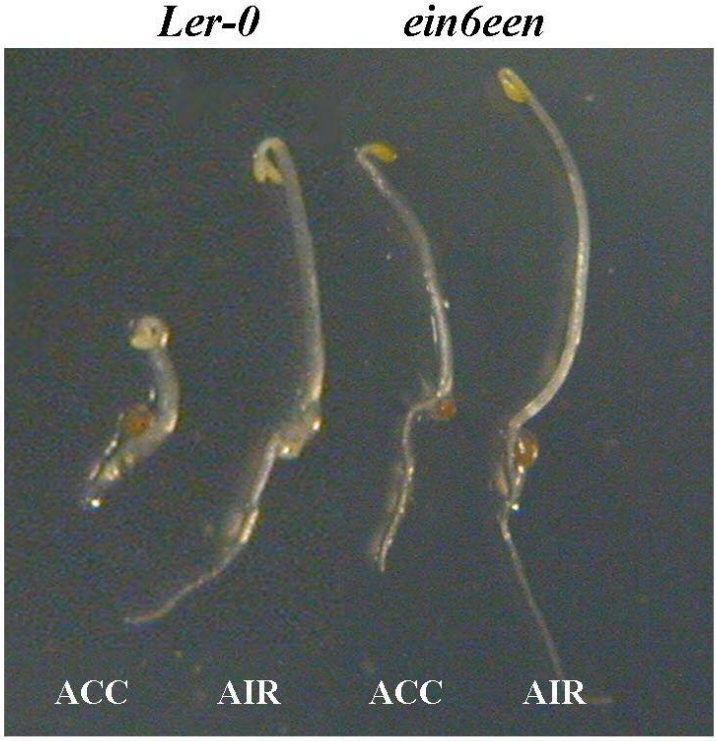
Table 2.2 continued

Marker	Primers	Type	Enzyme	Reference
T19P19	5'CATATAAGTAGAACTATATGGG3' 5'CGTGAATGGTTTTGTATATGC3'	SSLP	n/a	unpublished

Figure 2.1. Seedling Phenotypes of *ein6* and *ein6een* mutants

A. Phenotypes of wild-type (Ler-0) and mutant (*ein6een*) as seen grown in air or on 10μM ACC plates for three days in the dark.
B. Comparison of *ein6een* showing the ethylene insensitive phenotype (Ein^-) and *ein6-1* showing the ethylene insensitive root phenotype (Eir^-) to other ethylene insensitive mutants, from left to right *ein2-5*, *ein3eil1*, *ein3*, *ein6een*, *ein6-1*, wild-type (Col-0) and wild-type (Ler-0) grown on 10μM ACC plates for three days in the dark.

A.


Ler-0
ein6een
ACC
AIR
ACC
AIR

B.

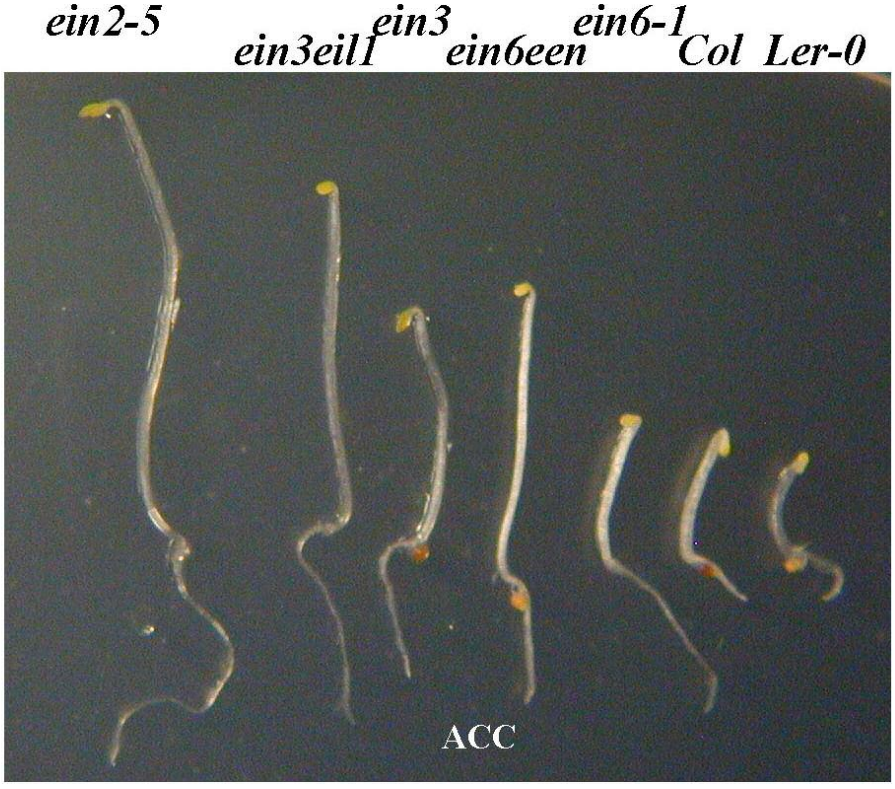
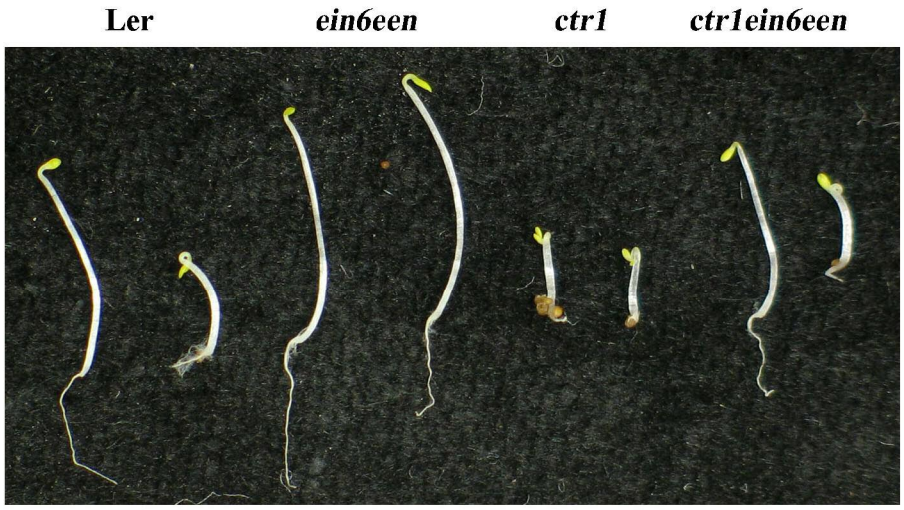

ein2-5
ein3eil1
ein3
ein6een
ein6-1
Col
Ler-0
ACC

Figure 2.2. Epistasis of *ein6een* to *ctr1*

A. Seedling phenotypes of wild-type Ler, *ein6een*, *ctr1* and the triple mutant of *ctr1ein6een* all grown on LS plates or LS supplemented with 10μM ACC (-ACC, +ACC respectively) for three days in the dark. The triple mutant combination shows a rescue of the severe *ctr1* phenotype to wild-type like growth in both –ACC and +ACC conditions.
B. Adult phenotypes of the same cross shown in (A), again showing the rescue of the severely dwarfed *ctr1* phenotype to a wild-type like phenotype in the triple mutant, *ctr1ein6een*.
C. *CTR1* expression in wild-type seedling tissues. *CTR1-GUS* construct in wild-type seedlings, showing ACC inducible expression in the apical hook region.
D. *CTR1* expression in *ein6een* seedling tissues. *CTR1-GUS* construct in *ein6een* seedlings showing a marked reduction in ACC inducible expression in the apical hook region.

A.


Ler
ein6een
ctr1
ctr1ein6een
-ACC +ACC -ACC +ACC -ACC +ACC -ACC +ACC

B.


ein6-1een
ctr1
ctr1ein6een

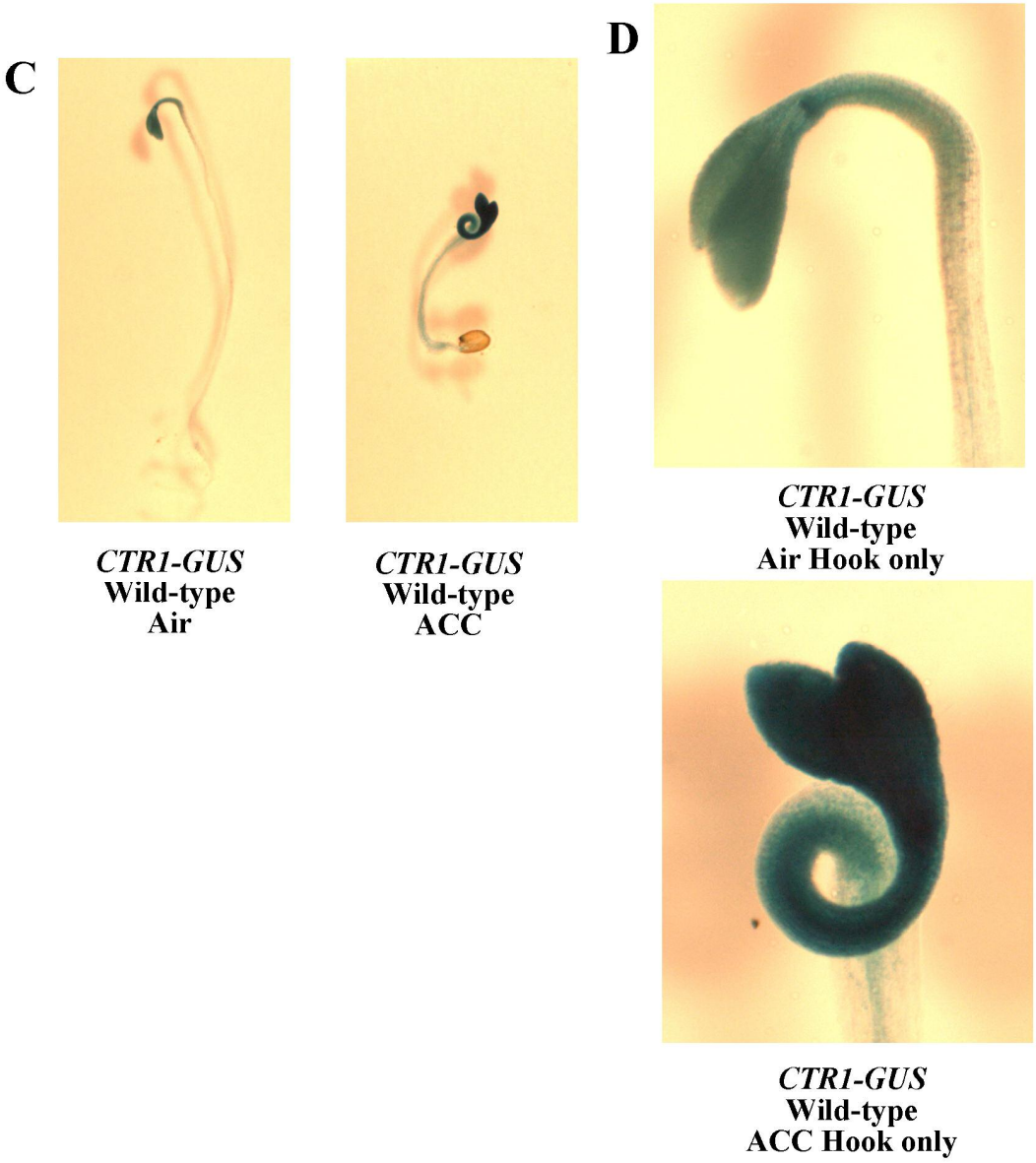


Figure 2.2 continued

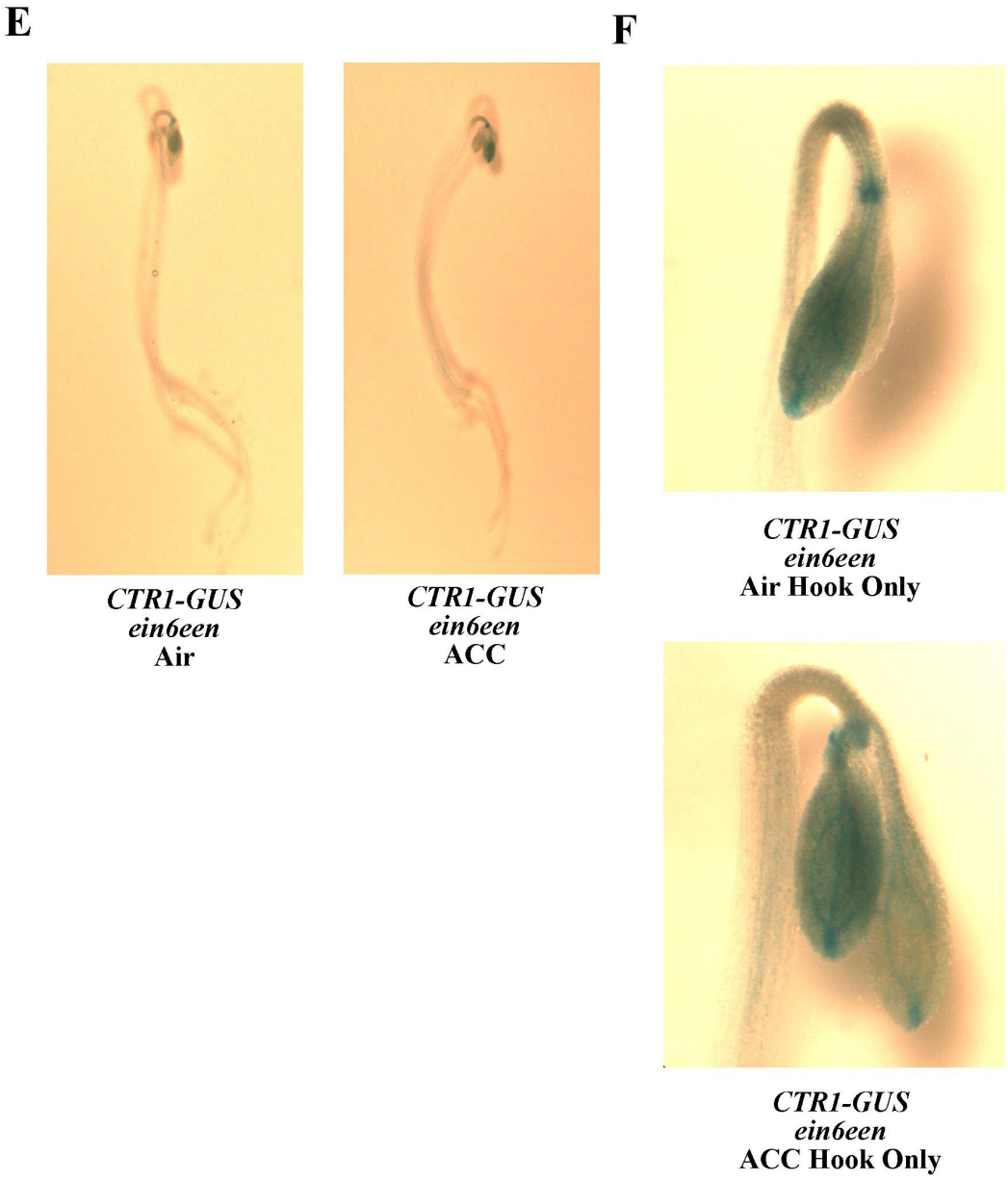
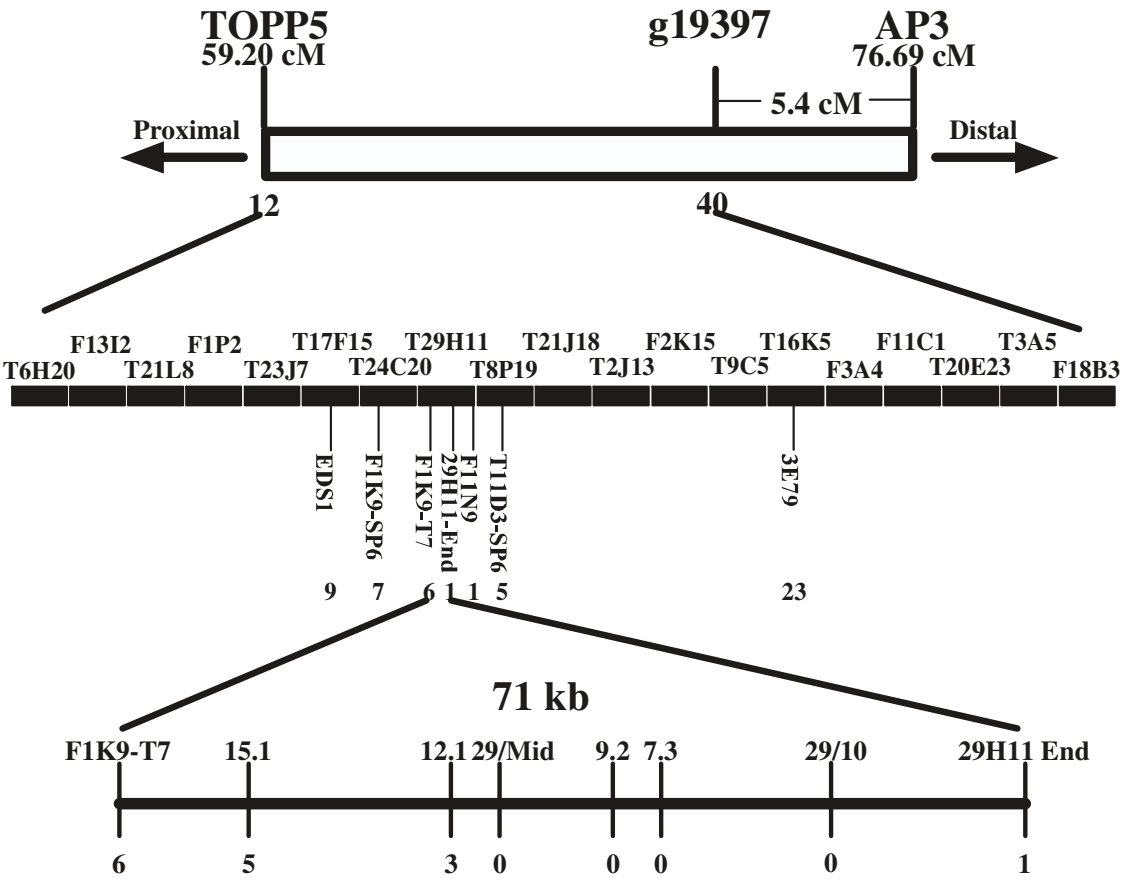


Figure 2.2 continued

Figure 2.3. Mapping and cloning of the *EIN6* locus

A. Map location of *EIN6* on Chromosome 3
B. *EIN6* gene intron-exon structure shown by boxes versus lines respectively, position of *ein6-1* mutation is noted
C. EIN6 protein domain structure, showing jumonji N, jumonji C and zinc finger domains along with the location of the *ein6-1* truncation
D. Alignment of JMJC protein domain as defined by ScanProsite (http://www.expasy.ch/tools/scanprosite/) between EIN6 and two human homologs that have been shown to have histone demethylase activity, JARID1A (RBP2) and JHDM3A. Black boxes show identical residues, grey boxes show conserved residues, blue and yellow boxes show sites that have previously been shown to be necessary for histone demethylase activity, blue for a-ketoglutarate binding activity and yellow for Fe (II) binding activity (Klose et al., 2006)
E. Complementation of *ein6* mutant phenotype, using the genomic DNA from the region containing At3g48430 and its promoter, the double mutant phenotype of *ein6een* plants is restored to a wild-type phenotype when grown on ACC

A.


TOPP5
59.20 cM
g19397
AP3
76.69 cM
5.4 cM
Proximal
Distal
12
40
F13I2
F1P2
T17F15
T29H11
T21J18
F2K15
T16K5
F11C1
T3A5
T6H20
T21L8
T23J7
T24C20
T8P19
T2J13
T9C5
F3A4
T20E23
F18B3
EDS1
F1K9-SP6
F1K9-T7
29H11-End
F11N9
T11D3-SP6
3E79
9
7
6
1
1
5
23
71 kb
F1K9-T7
15.1
12.1
29/Mid
9.2
7.3
29/10
29H11 End
6
5
3
0
0
0
0
1

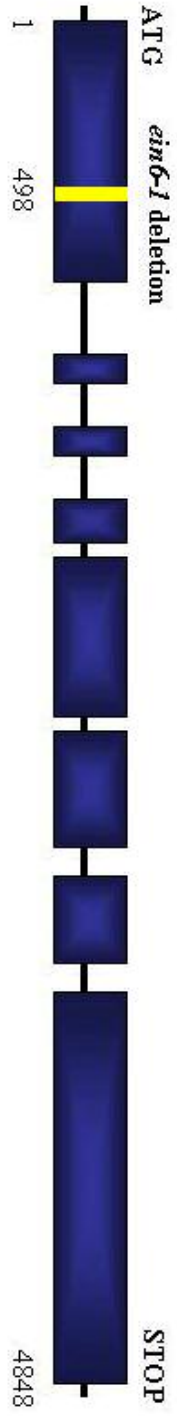


Figure 2.3 continued
B.

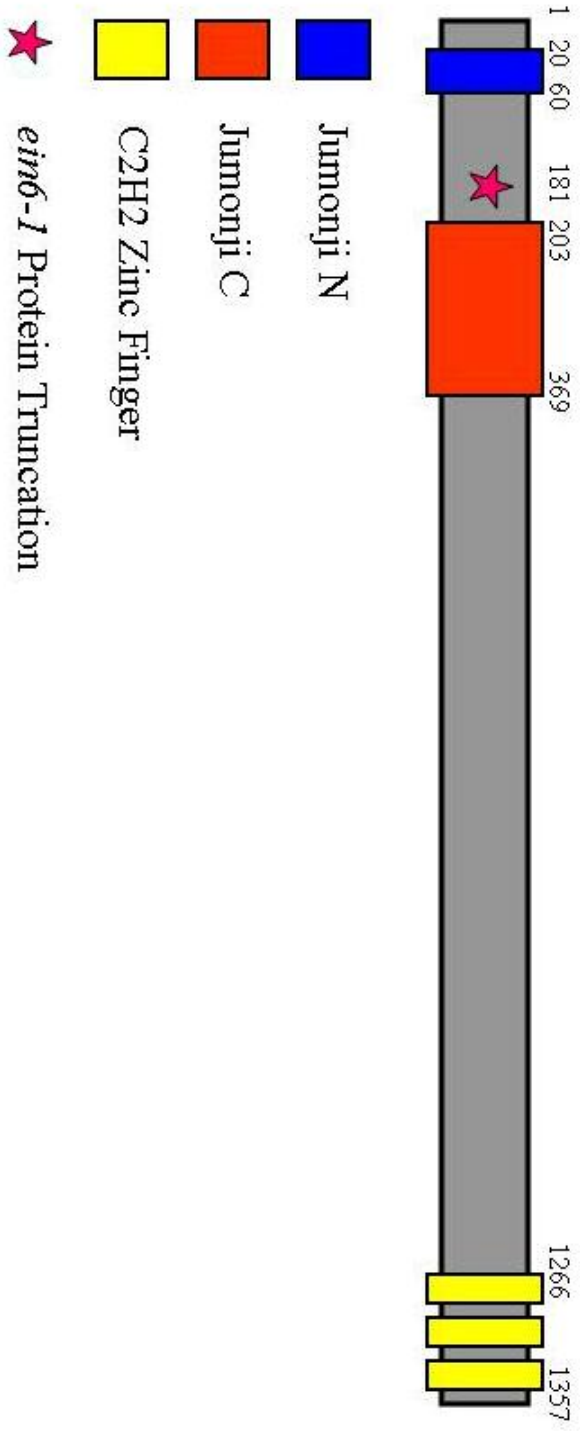


Figure 2.3 continued
C.

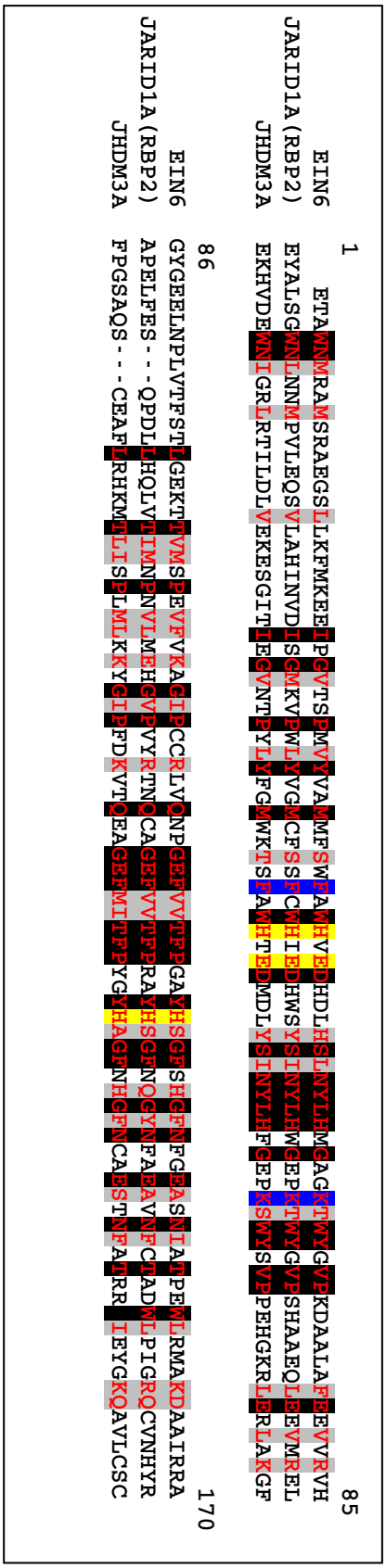
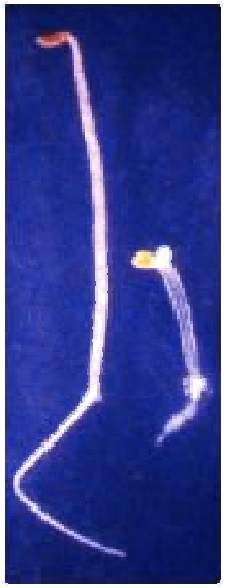


Figure 2.3 continued
D.

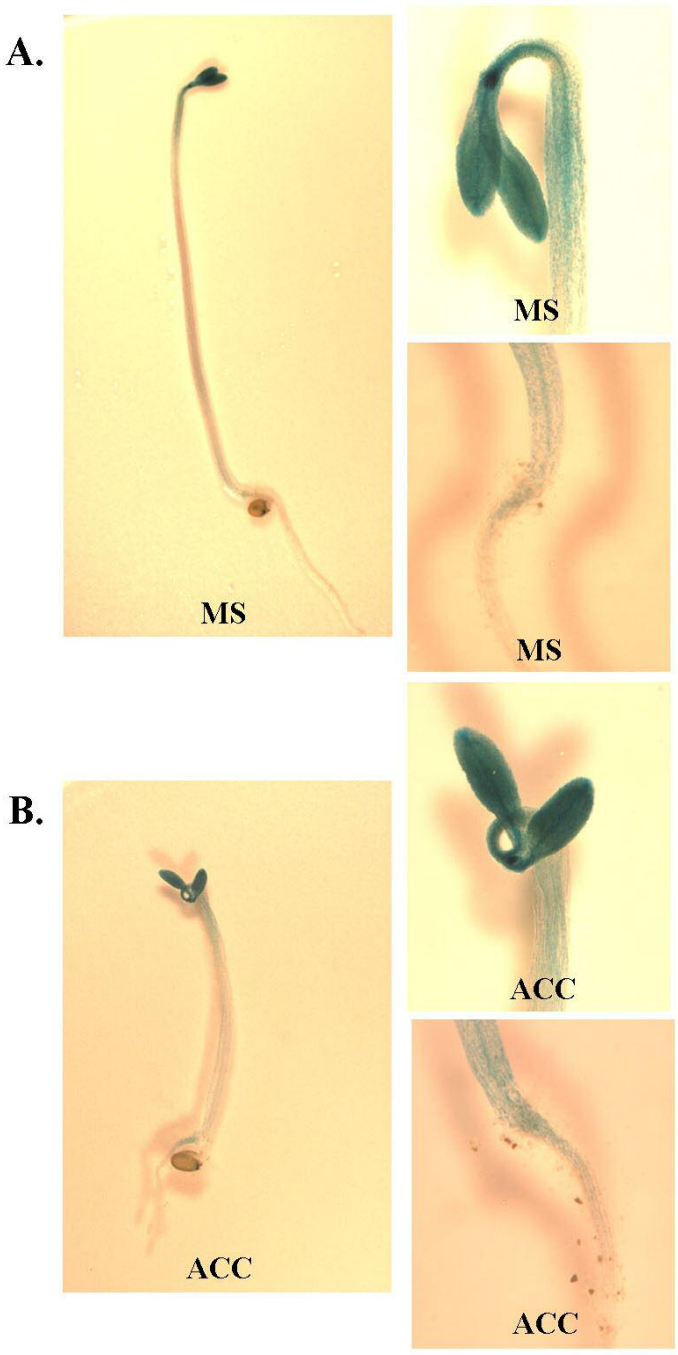
ein6/een +
empty vector

ein6/een +
EIN6 genomic fragment



+ 10μm ACC

Figure 2.3 continued
E.



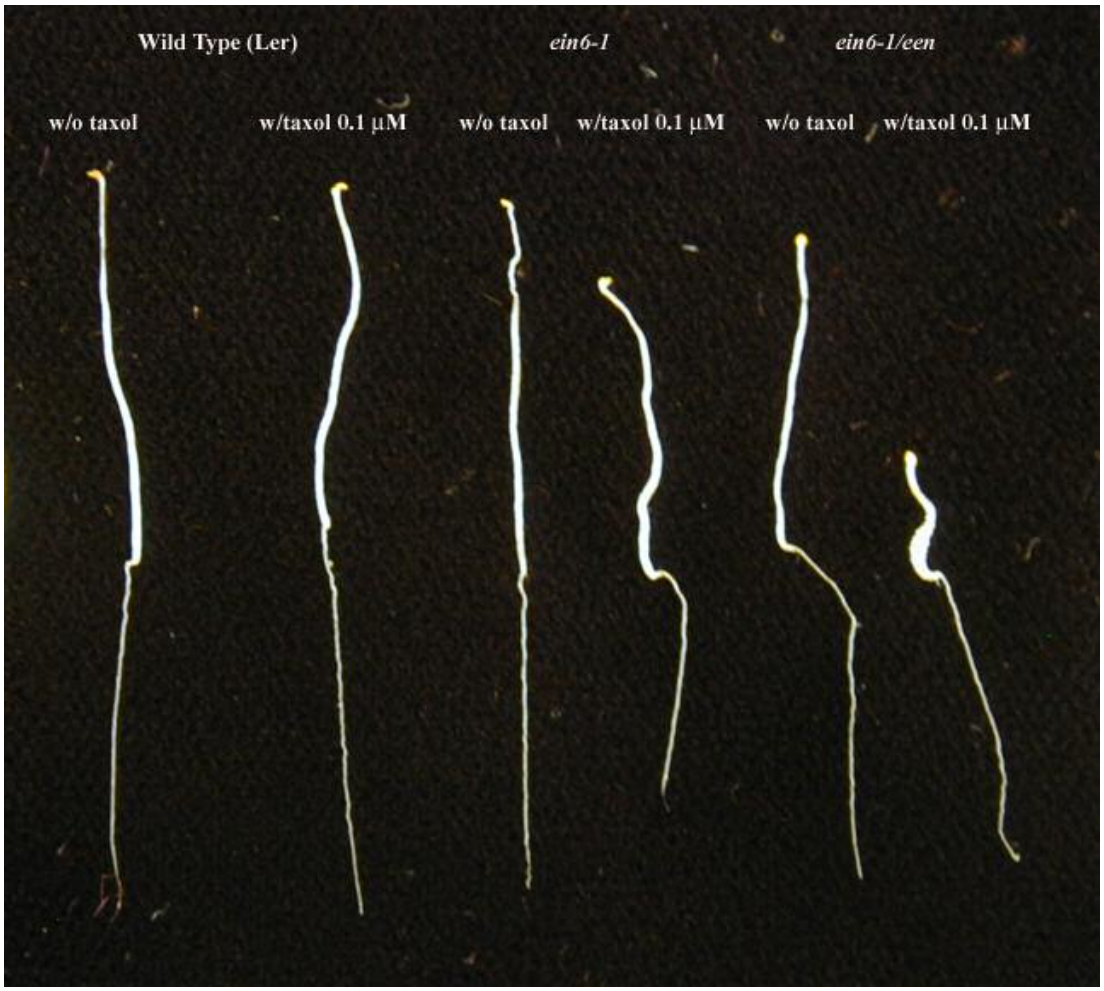
Chapter 2 Figure 4. Expression of EIN6

A. *EIN6-GUS* in wild-type seedlings and enlargement of the hook and hypocotyl root junction, grown for three days in the dark in air
B. *EIN6-GUS* in wild-type seedlings the presence of the ethylene precursor ACC

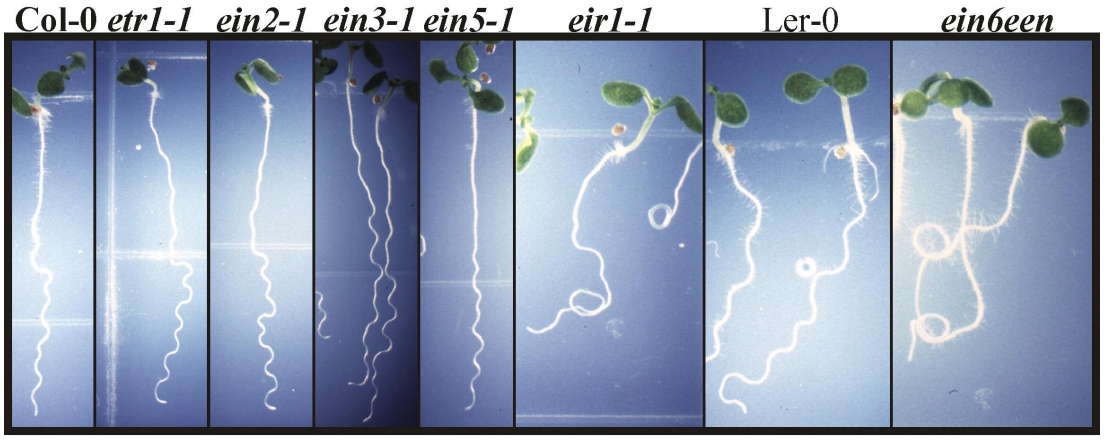
Figure 2.5. Non-ethylene related phenotypes of *ein6een* mutants

A. Hypersensitivity to the microtubule stabilizing agent taxol. Showing wild-type Ler, *ein6-1* and *ein6een* seedlings grown with and without the drug taxol at a 0.1μM concentration for 10 days in the dark.
B. Obstacle avoidance root waving assay, ethylene insensitive mutants show normal gravitropic response with the exception of *eir1* and *ein6een* both showing an agravitropic response.
C. Trichome branching defect. Trichomes from adult leaves of wild-type Ler, *ein6-1* and *ein6een* grown in long day growth conditions (see methods).

A.


Wild Type (Ler)
ein6-1
ein6-1/een
w/o taxol
w/taxol 0.1 µM
w/o taxol
w/taxol 0.1 µM
w/o taxol
w/taxol 0.1 µM

B.


Col-0
etr1-1
ein2-1
ein3-1
ein5-1
eir1-1
Ler-0
ein6een

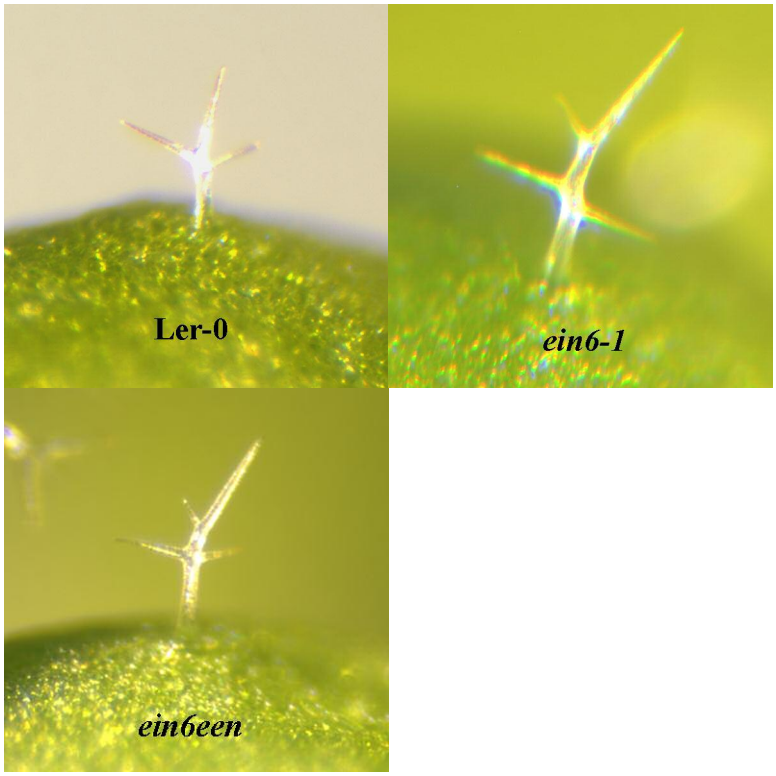


Figure 2.5 continued
C.

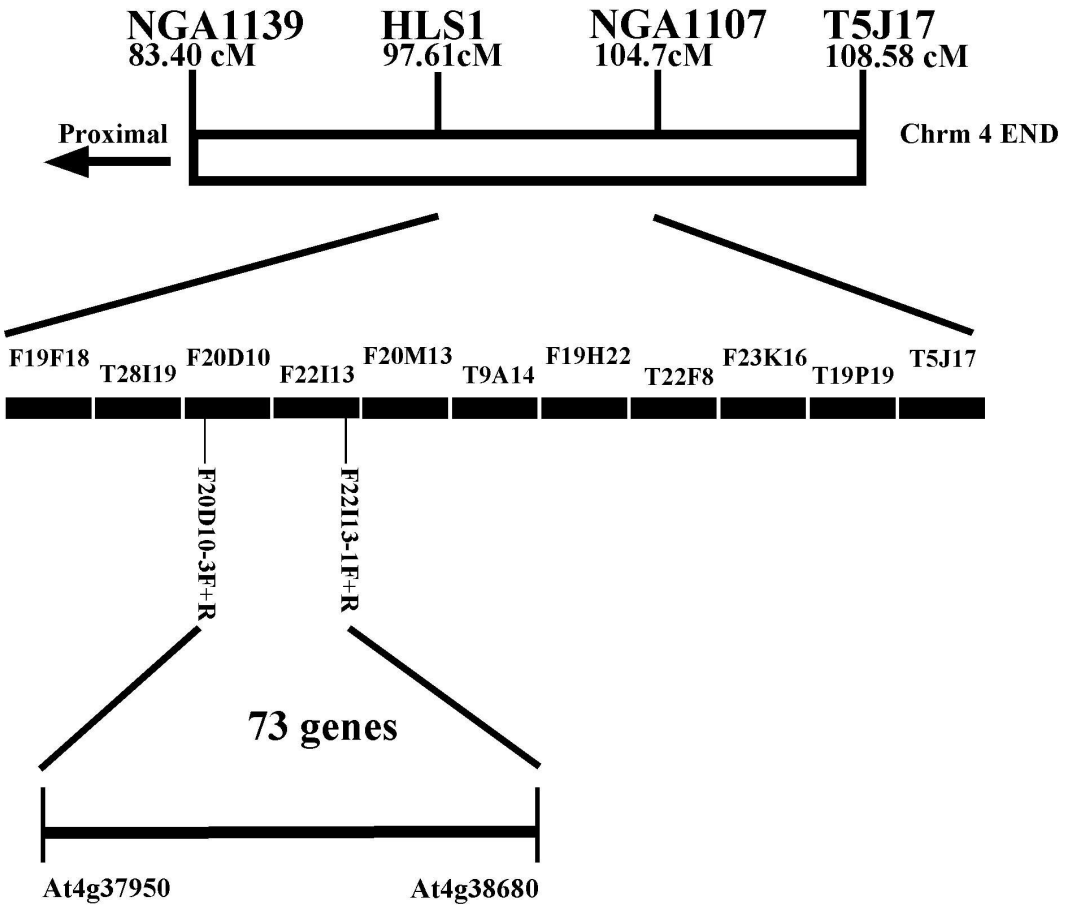


Figure 2.6. Chromosomal location of the *een* mutation.

een mapped to a region of 73 genes at the end of Chromosome 4 between BACs F20D10 and F22I13.

Chapter III

Mapping the *ENHANCER OF ETHYLENE INSENSIVITY*

SUMMARY

The *ENHANCER OF ETHYLENE INSENSITIVITY*, *een*, was found as the first enhancer of the ethylene signaling pathway in *Arabidopsis*. The mutant enhances the ethylene insensitive root phenotype of *ein6-1*. The identification of this mutant is of great importance to understanding how the ethylene signal is transduced and how ethylene relates to other processes in the cell. Various methods, including Array Mapping, map based cloning and complementation, have been undertaken to determine the nature of the *een* mutation. Although the locus has been narrowed to a small region of genes, the exact character of the mutation has not been discovered.

BACKGROUND

The *ENHANCER OF ETHYLENE INSENSITIVITY* was discovered in a screen for seedlings that failed to exhibit the normal triple response to ethylene (Roman et al., 1995). It occurred as a second simultaneously mutation in the mutant *ethylene insensitive six*, *ein6*, which enhanced the phenotype of *ein6* from an ethylene insensitive root mutant, Eir^{-}, to an ethylene insensitive mutant, Ein^{-}. The mutagen used to generate this mutant seed was fast-neutron irradiation. Fast neutron irradiation generally induces chromosomal breaks resulting in deletions ranging from a few base pairs to multiple kilobases (Shirley et al., 1992). The segregation frequency observed in mutants generated by fast neutron irradiation is lower then the expected Mendelian ratio in 18.1% of mutants generated (Dellaert, 1980). This may be a result of reduced gametophitic transmission of the mutation or of multiple simultaneous mutations existing in the mutant background. The example of a weak mutation and a genetic enhancer or modifier generated by the same mutagenesis has also been previously characterized as in the study by Ohshima and colleagues on *Arabidopsis* genes required for tobacco mosaic virus infection, which identified a recessive mutation *tom2* and a semi-dominant modifier *ttm1* (Ohshima et al., 1998).

een is the first enhancer mutant found for the ethylene signaling pathway. Their have been numerous reverse genetic screens for suppressors of ethylene insensitivity, which have identified new components of the signal transduction pathway, such as the suppressor of the dominant ethylene receptor allele *etr1*, *REVERSION TO ETHYLENE-SENSITIVITY* (Resnick et al., 2006). However, their

have not been genetic screens for enhancers of the ethylene response, because the phenotypic response is fairly saturating in most mutant seedlings with Ein$^-$ mutants demonstrating maximal hypocotyl elongation and *enhanced ethylene response*, Eer$^-$, mutants demonstrating minimal hypocotyl elongation. It would be possible to conduct these screens on less severe ethylene mutants such as the *weak ethylene insensitive*, *ein3 like* families of mutants.

The double mutant *ein6een* shows disruption in normal ethylene responses. This is demonstrated phenotypically by its ethylene insensitivity. There is also a requirement for these two mutants in accumulation of EIN3 protein levels, where EIN3 protein accumulation is greatly decreased in *ein6een*, just as is seen for *ein2* (Guo and Ecker, 2003). Additionally *ein6een* shows a number of novel microtubule related defects that have not been characterized for other ethylene insensitive mutants. The gene for *EIN6* has been cloned and appears to encode a protein that may act as a histone H3 lysine K4 demethylase. There have not been any previously identified histone demethylases in plants. Methylation on H3K4 is a mark of actively transcribed DNA (euchromatin) (Martin and Zhang, 2005). The non-plant eukaryotic organisms that share the greatest degree of protein similarity to EIN6 have been biochemically characterized as specific demethylases of trimethylated H3K4 (Klose et al., 2007). Progress is underway to determine whether EIN6 shares this biochemical function with its homologs (Colangelo and Ecker personal communication). Identification of the nature of the *een* mutation should shed light on a link between

downstream ethylene responses and regulation of transcription by chromatin remodeling.

RESULTS and DISCUSSION

Mapping of the *EEN* locus was not concurrent with mapping of the *EIN6* locus. Initial mapping employed two techniques, bulk segregate analysis using Affymetrix GeneChip© technology and traditional map based cloning. This type of bulk segregate analysis was first described by Hazen and colleagues also using mutants generated by fast neutron irradiation (Hazen et al., 2005). For the bulk segregate analysis the same mapping population was used that was generated for map based cloning, *ein6een* plants crossed to Col-0. F2 seedlings were selected by phenotype Ein^- or Ein^+ and pools of approximately 100 of each group were made. DNA was prepared from each of these pools, labeled using Invitrogen, BioPrime DNA Labeling Kit® and hybridized to Affymetrix ATH1 microarrays. Full details of this protocol can be found at http://naturalsystems.uchicago.edu/naturalvariation/methods/. A graphical view of the results of the array mapping can be seen in Figure 1. Linkage was found to two areas of the genome, to chromosome 3, the location of the *EIN6* locus and to the end of chromosome 4, the presumed location of the *EEN* locus. There were no areas of probes that lacked hybridization across chromosome 4, indicating that the mutation causing *een* is probably not a gross deletion, although this analysis is limited by the probe distribution on the Affymetrix ATH1 microarrays.

Using the same mapping population as for bulk segregate analysis, of *ein6een* plants crossed to Col-0, initial mapping analysis revealed the same two areas of linkage in this population using 26 SSLP markers spread along the 5 chromosomes of *Arabidopsis* (Bell and Ecker, 1994) as was found in the array mapping. The first area

of linkage corresponded to the location of the *EIN6* locus on the short arm of chromosome 3. The second area of linkage corresponded to the markers NGA 1139 and NGA 1107, located near the end of chromosome 4. Additional SSLP markers were designed from repeat sequence from Ler and Col, and narrowed the location of the mutation to a 73 gene region on BACs F20D10 and F22I13. Figure 2 shows a schematic representation of the mapping region at the end of chromosome 4.

Concurrently with map based cloning an additional strategy for isolating the location of the *EEN* locus by complementation was undertaken. This strategy involved the use of transformable artificial chromosomes, TACs, transformed into *ein6een* plants to complement the mutant phenotype. This approach has been used successfully in identification of *Arabidopsis* mutants in the past (Xiong et al., 2001). The TAC library selected was the John Innes Center JAtY clone library consisting of 36,864 clones made from *Arabidopsis* Col-0 leaf DNA. These clones were constructed using the pYLTAC7 vector and have an average insert size of 80kb. 10 clones that covered the rough mapping region were selected based on the available end sequences of the clone. These clones overlapped to allow for the most complete coverage of the mapping region. There was however one gap in the coverage, which did fall into the final mapping region of 73 genes. These clones were transformed into *ein6een* adult plants, the T1 plants were screened for BASTA resistance, segregating T2 plants were scored for phenotype Ein^-, no complementation, or Ein^+, complementation, and these plants were again scored for BASTA resistance to segregate with phenotype. One clone out of the 10 showed a complemented

phenotype that segregated with BASTA resistance (Figure 3). This clone, 67N20, contained a region of the chromosome that has 29 putative genes and fell within the final mapping region of 73 genes. After additional transformations were tested a second clone, 70D11, also complemented the Ein$^-$ phenotype. This second clone overlapped the first clone 67N20 by nine genes. A schematic view of the region can be seen in Figure 2. A listing of the nine genes and their functions can be found in Table 3.1.

There has been little published about these 9 final genes in the region of complementation. Seven of the nine have putative functions assigned based upon protein homology to genes of known function. Of the remaining two, one is an extremely large gene, *KAKTUS1*, *KAK1*, encoded by a nearly eight kilobase genomic region with 17 exons (El Refy et al., 2003). Alleles of this mutant have been characterized and have a supernumerary trichome branching phenotype. Although this is a phenotype also seen in *ein6een* mutants, the *kak1* mutant have a much more severe phenotype. The entire genomic region for *KAK1* is not on the clone 67N20, but there are known splice variants of the gene. The other published gene is a putative farnesylated protein, *ATFP6*, which was found to be a gene upregulated by heat acclimation (Lim et al., 2006), there are no reported mutants of this gene. Of the unpublished genes in the region all of them could be implicated in hormone related function based upon previous studies with genes of related function. Three of the genes fall into the same functional category, putative phospholipases. Another candidate is the putative protein phosphatase 2C, Arabidopsis protein phosphatases

have been associated with control of phosphorylation state in multiple hormone signaling pathways including PP2C in abscisic acid (ABA) signaling (Meyer et al., 1994) and PP2A in both auxin and ABA signaling (Garbers et al., 1996; Kwak et al., 2002). Hydrogen ATPases have also been related to auxin transport (Li et al., 2005) and to regulation of osmotic stress (Beffagna et al., 2005). There is also a monooxygenase in the final candidates, Arabidopsis flavin monooxygenase-like enzymes have been found to have function in the auxin pathway (Zhao et al., 2001) and also in defense responses (Mishina and Zeier, 2006). Finally the last gene in the region is a phosphatidylinositol synthase, a chip based study found that phosphatidylionositol signaling pathways were regulated by multiple hormone treatments (Lin et al., 2004).

A candidate gene approach clearly will not work for isolating the *EEN* locus from the final group of contenders due to the fact that all of the genes could be associated with hormone signaling. Since there were no gross deletions seen in this region, as seen by DNA hybridization to Affymetrix ATH1 microarrays, the mutation is probably similar in nature to that of the *ein6-1* mutation, a small deletion. Complications with identification of the specific Ler parental ecotype of the *ein6een* mutants have arisen because the mutant was not generated in the Ecker lab; therefore sequencing has also not been a viable method for identification of the mutation. It appears that further complementation is the only feasible means of confirmation of the *EEN* locus. This approach is being applied using digestions of the BAC F20M13. Size selected fragments of the BAC are cloned into the vector pMN19, which contains

four copies of the CaMV 35s promoter and was used by Weigel and colleagues to introduce genomic fragments into *Arabidopsis* in order to recapitulate activation tag mutant phenotypes (Weigel et al., 2000). This approach should allow for the exact nature of the *een* mutation to be revealed.

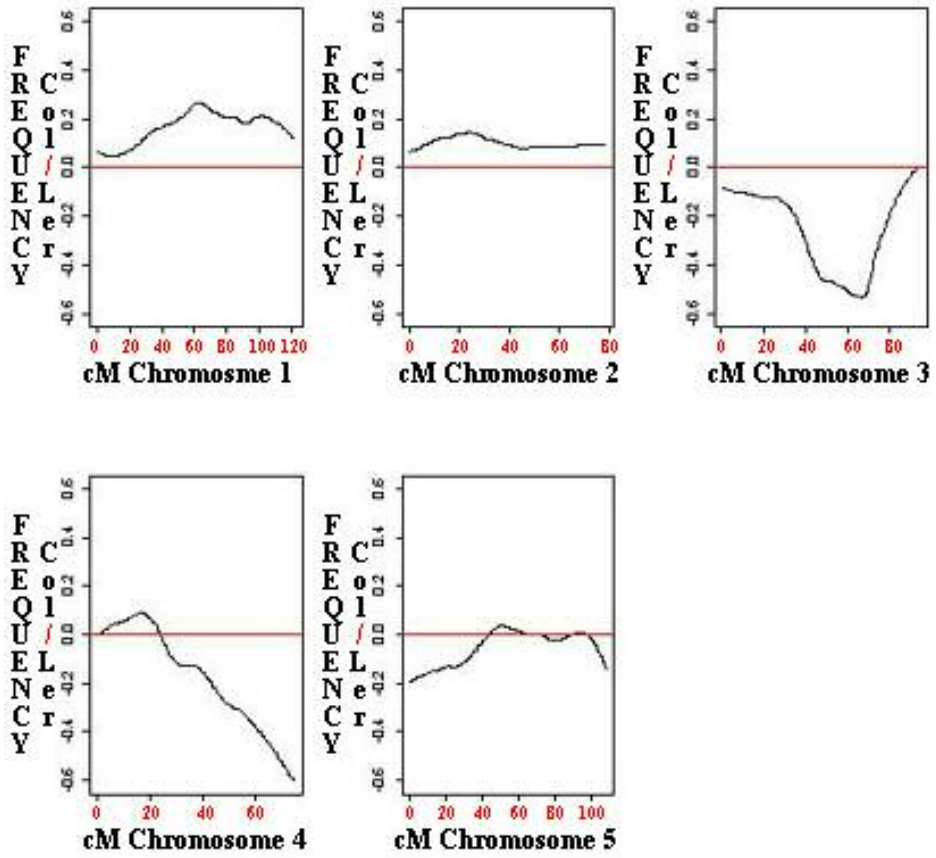


Figure 3.1. Bulk segregate mapping of *EEN* locus.

Each box represents a chromosome of Arabidopsis, where the X-axis is equal to distance in cM across the chromosome and the Y-axis represents the ratio of % Col DNA to % Ler DNA. When the black line moves from the center red line towards Col (top) or Ler (bottom) this represents linkage to that accession in that region of the chromosome.

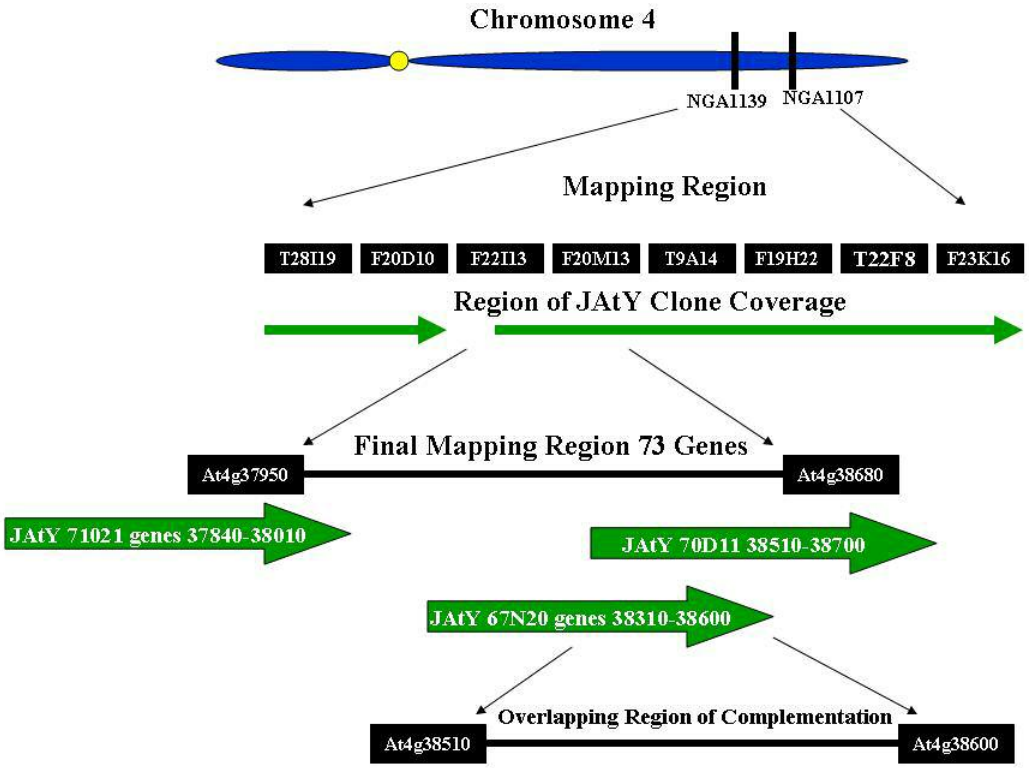


Figure 3.2. Chromosome 4 region of *een* mapping.

From the top SSLP markers showing linkage, BACs in mapping region, BACs covered by JAtY TAC clones, final mapping region as defined by SSLP mapping, Overlapping JAtY clones within final mapping region, region of complementation by JAtY clones (see Table 1 for list of genes).

Figure 3.3. Complementation of ethylene insensitivity with JAtY clone 67N20.

Seedlings grown for three days in the dark on 10μM ACC, on the left a segregating non-complemented seedling and on the right a complemented *ein6een* seedling segregating kanamycin resistance with the complementation phenotype.

Table 3.1. Genes in final overlapping region of JAtY complementation for *EEN* gene

Gene #	**At ID**	**Putative Gene Function (by BLAST)**
Gene 1	At4g38510	Hydrogen exporting ATPase
Gene 2	At4g38520	Protein Phosphatase 2C
Gene 3	At4g38530	Phospholipase C
Gene 4	At4g38540	Monooxygenase 2
Gene 5	At4g38550	Phospholipase like (expressed protein)
Gene 6	At4g38560	Phospholipase like (expressed protein)
Gene 7	At4g38570	Phosphatidylinositol synthase
Gene 8	At4g38580	FARNESYLATED PROTEIN 6 Metal ion binding
Gene 9	At4g38590	BGAL14 Putative beta-galactosidase
Gene 10	At4g38600	KAKTUS Ubiquitin ligase

CHAPTER IV

Conclusions

A MODEL FOR EIN6 AND EEN IN ETHYLENE SIGNAL TRANSDUCTION

Two simultaneous mutations have been isolated which cause an ethylene insensitive seedling phenotype, implicating their requirement for proper ethylene signaling in the model plant, Arabidopsis thaliana. These mutations *ethylene insensitive six* (*ein6*) and the *enhancer of ethylene insensitivity* (*een*) have been phenotypically, genetically and biochemically linked to the ethylene signal transduction pathway and are genetically located downstream of the negative regulator of ethylene signaling, CTR1. A mutation in *ein6* alone confers a mild insensitivity to exogenous ethylene treatment, mainly conveyed as an ethylene insensitive root phenotype. Single mutants in *een* do not have any noticeable phenotypic differences from wild-type plants. Both of these genes are required for the ethylene induced accumulation of the primary ethylene transcription factor EIN3, as the double mutant combination does not accumulate EIN3 protein with exogenous ethylene gas treatment. A mutation in *ein6* alone confers a mild insensitivity to exogenous ethylene treatment, mainly conveyed as an ethylene insensitive root phenotype. Single mutants in *een* do not have any noticeable phenotypic differences from wild-type plants.

In addition to the ethylene insensitivity conferred by these two mutations they also have a number of other ethylene independent phenotypes. The other class of phenotypes displayed by these two mutations can be generally characterized as microtubule related phenotypes. These phenotypes include a defect in the root waving assay, a hypersensitivity to the drug taxol and supernumerary trichome branching. Although the combination of the microtubule phenotypes seen in these mutants has

not been previously reported they all support a role of the *ein6een* mutant combination in the stability of microtubules and more specifically in their destabilization. This is not however completely unexpected as the re-orientation of cortical microtubules has long been thought to be a downstream effect of ethylene signaling (Apelbaum and Burg, 1971). Further support for the relationship between ethylene and microtubules comes from a study of another ethylene related mutant that shows nearly converse microtubule phenotypes to those demonstrated by *ein6een*, a mutation in the locus encoding the *POLARIS* (*PLS*) peptide (Casson et al., 2002; Chilley et al., 2006).

In addition to the clues given by the characterization of these mutants phenotypically and genetically, hypotheses on the functions of the protein that encoded by the *EIN6* locus also allow us to predict its role. Their have been many functional characterizations of orthologs of EIN6 in non-plant systems (Klose et al., 2006; Lee et al., 2007; Liang et al., 2007). Recent work has uncovered that these proteins may act as histone demethylase enzymes, altering the epigenetic mark of histone methylation which had been though to be a permanent epigenetic mark. Histone methylation is one form of covalent modification of the histone tails of the core histones, H2A, H2B, H3 and H4. In addition to methylation other types of modification of histone tails include: acetylation, phosphorylation sumoylation, ubiqutination and ADP ribosylation. Covalent modification of the core histones results in the "histone code" which in combination with other modifications such as DNA methylation results in changes in transcription of target genes (Jenuwein and Allis, 2001). Histone methylation can occur on either arginine or lysine residues.

There can be different levels of histone methylation, with residues being modified to a mono-, di- or trimethylated state. Each mark, depending on which histone it is modifying and which level of methylation occurring, conveys a different message about the transcriptional state of the gene encoded by the DNA around that histone. For instance methylation of histone H3 on lysine 4, 36 or 79 is a mark of active or euchromatic DNA, while a mark on histone H3 at lysine 9 or 27 or H4 at lysine 20 indicates a mark of silenced or heterochromatic DNA.

The first histone demethylase enzyme that was discovered was LSD1 (Shi et al., 2004), a well conserved protein found throughout species ranging from yeast to humans, including four family members in *Arabidopsis thaliana*. This protein was biochemically characterized as a histone demethylase and was found to specifically demethylate Histone H3 lysine 4 (H3K4). Recent work has emerged on an additional family of histone demethylase enzymes (Klose et al., 2006). These proteins all contain Jumonji domains and have been found to demethylate various methylated histones (H3 and H4) and to be specific for a number of levels of histone modification (mono-, di-, and trimethylated forms). The subfamily that is most closely related to EIN6 is the JARID1 family (see Chapter 2 figure 3, panel D)(Klose et al., 2006). EIN6 contains the conserved residues for α-ketoglutarate and ferrous iron binding that have been found to be essential for enzymatic activity of this protein family. Other members of the JARID1 family have been found to act as H3K4 di- and trimethyl demethylases specifically *in vivo*(Seward et al., 2007). This demethylase then acts to remove the mark of active or euchromatic DNA and would therefore result in the

silencing of the gene associated with the demethylated histone. Mutations in an H3K4 specific demethylase would result in constitutive activation of target genes as the mark of activation would not be able to be removed.

All the evidence both from experimental procedures and from a hypothesis of protein function leads to a model for how *EIN6* and *EEN* may be functioning in the ethylene signal transduction pathway. *EIN6* and *EEN* are genetically downstream of *CTR1*. For the ethylene pathway to signal properly both genes need to have intact function. A mutation in *ein6* alone does not impair signaling through EIN3. Mutations in *ein6* and to a greater extent in the double mutant *ein6een* have effects on the stability of microtubules, which may be a downstream end product of ethylene signaling. Functional analysis of the EIN6 protein will confirm if it is acting as an H3K5 di- or tri- histone demethylase acting to remove the mark of active DNA. If the EIN6 protein is able to function in this manner, mutants in the gene would result in a higher overall level of histone methylation and a higher overall level of active genes in the mutant. Targets of the EIN6 protein could include cytoskeletal genes such as α and β tubulins, which when constitutively activated could cause the microtubule related phenotypes of this mutant. Although the specific identity of the locus associated with the *een* mutant has not been identified, it seems as though this gene has an effect on the post-transcriptional regulation of EIN3. Perhaps this gene functions by regulating EIN3 protein on the level of the *EBF1/2* genes or the *EIN5* gene which all play a role in controlling EIN3 protein levels (see Chapter IV Figure 1 for a schematic representation).

Future Directions

To further clarify the role of these two genes in the ethylene signal transduction pathway further experiments are needed. Characterization of the EIN6 protein, including whether/how it acts as a histone demethylase is central to the future of this project. The characterization of the EIN6 protein may include identification of interacting proteins by a yeast two hybrid assay. Additionally the production of an EIN6 antibody would allow for further biochemical experiments to be conducted. These biochemical experiments could include using the EIN6 antibody to pull down physically interacting proteins and identification of these proteins by Mass Spectrometry. Another biochemical assay to determine if the protein does indeed act as a histone demethylase would be to use either a full or partial fragment of the EIN6 protein in the same assays conducted by the other groups working on Jmj histone demethylases to see if it has the enzymatic activity of a histone demethylase and which mark and level of histone demethylation it is specific for. Determining whether overall histone methylation is changed in the *ein6* or *ein6een* mutants could be carried out by western blot using histone methylation specific antibodies. If EIN6 is found to be a histone demethylase chromatin immunoprecipitation (chIP), using an antibody specific for its level and mark of demethylation activity, hybridized to a microarray (chip) would be helpful in identification of the specific genome wide targets of the protein. Additionally determining the locus associated with the *een* mutation will be crucial in uncovering how these two genes act together to cause an ethylene insensitive phenotype.

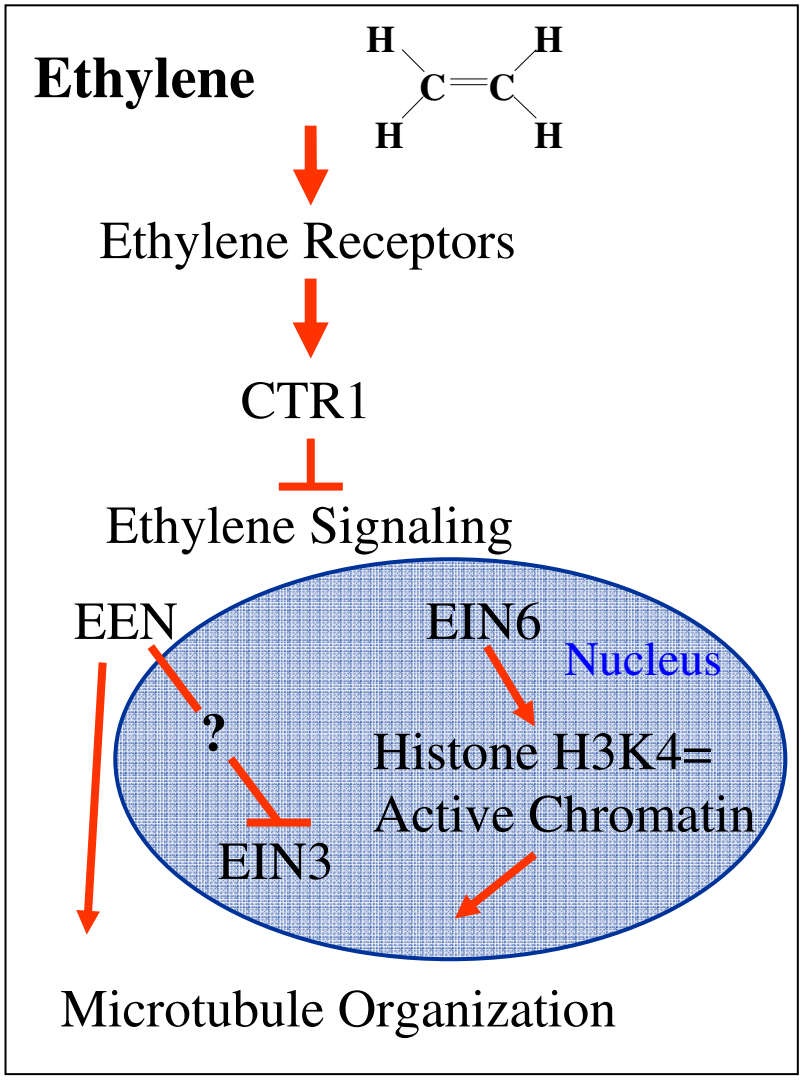


Figure 4.1. A model for EIN6 and EEN in ethylene signal transduction

EIN6 and EEN function downstream of the negative regulator of ethylene signaling, CTR1. EEN controls the levels of EIN3 protein through an unknown mechanism, while EIN6 may function as a histone demethylase. Mutations in both genes effect the function of both proper ethylene signal transduction and cellular microtubule organization.

REFERENCES

Abel S, Nguyen MD, Chow W, Theologis A (1995) ACS4, a primary indoleacetic acid-responsive gene encoding 1- aminocyclopropane-1-carboxylate synthase in Arabidopsis thaliana. Structural characterization, expression in Escherichia coli, and expression characteristics in response to auxin [corrected]. J Biol Chem **270:** 19093-19099.

Abeles FB, Morgan PW, Saltveit ME (1992) Ethylene in Plant Biology, Vol 2. Academic Press, San Diego

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

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

Alonso JM, Stepanova AN, Solano R, Wisman E, Ferrari S, Ausubel FM, Ecker JR (2003) Five components of the ethylene-response pathway identified in a screen for weak ethylene-insensitive mutants in Arabidopsis. Proc Natl Acad Sci U S A **100:** 2992-2997.

Apelbaum A, Burg SP (1971) Altered Cell Microfibrillar Orientation in Ethylene-treated Pisum sativum Stems. Plant Physiol **48:** 648-652

Ault GS, Ryschkewitsch CF, Stoner GL (1994) Type-specific amplification of viral DNA using touchdown and hot start PCR. J Virol Methods **46:** 145-156

Bao Y, Kost B, Chua N-H (2001) Reduced expression of a-tubulin genes in Arabidopsis thaliana specifically affects root growth and morphology, root hair development and root gravitropism. The Plant Journal **28:** 145-157

Barlow JN, Zhang Z, John P, Baldwin JE, Schofield CJ (1997) Inactivation of 1-aminocyclopropane-1-carboxylate oxidase involves oxidative modifications. Biochemistry **36:** 3563-3569

Baskin TI, Wilson JE, Cork A, Williamson RE (1994) Morphology and Microtubule Organization in Arabidopsis Roots Exposed to Oryzalin or Taxol. Plant Cell Physiol. **35:** 935-942

Beffagna N, Buffoli B, Busi C (2005) Modulation of Reactive Oxygen Species Production During Osmotic Stress in Arabidopsis thaliana Cultured Cells: Involvement of the Plasma Membrane Ca2+-ATPase and H+-ATPase. Plant Cell Physiol. **46:** 1326-1339

Bell CJ, Ecker JR (1994) Assignment of 30 microsatellite loci to the linkage map of Arabidopsis. Genomics **19:** 137-144

Berrocal-Lobo M, Molina A, Solano R (2002) Constitutive expression of ETHYLENE-RESPONSE-FACTOR1 in Arabidopsis confers resistance to several necrotrophic fungi. Plant J **29:** 23-32.

Bleecker AB (1999) Ethylene perception and signaling: an evolutionary perspective. Trends Plant Sci **4:** 269-274.

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

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

Bokros CL, Hugdahl JD, Hanesworth VR, Murthy JV, Morejohn LC (1993) Characterization of the reversible taxol-induced polymerization of plant tubulin into microtubules. Biochemistry **32:** 3437-3447

Braam J, Davis RW (1990) Rain-, wind-, and touch-induced expression of calmodulin and calmodulin-related genes in Arabidopsis. Cell **60:** 357-364

Buer CS, Wasteneys GO, Masle J (2003) Ethylene Modulates Root-Wave Responses in Arabidopsis. Plant Physiol. **132:** 1085-1096

Burg SP, Burg EA (1967) Inhibition of polar auxin transport by ethylene. Plant Physiol **42:** 1224-1228.

Cancel JD, Larsen PB (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.

Casson SA, Chilley PM, Topping JF, Evans IM, Souter MA, Lindsey K (2002) The POLARIS gene of Arabidopsis encodes a predicted peptide required for correct root growth and leaf vascular patterning. Plant Cell **14:** 1705-1721

Chae HS, Faure F, Kieber JJ (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.

Chae HS, Kieber JJ (2005) Eto Brute? Role of ACS turnover in regulating ethylene biosynthesis. Trends Plant Sci **10:** 291-296

Chang C, Stadler R (2001) Ethylene hormone receptor action in Arabidopsis. Bioessays **23:** 619-627.

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

Chen W, Provart NJ, Glazebrook J, Katagiri F, Chang HS, Eulgem T, Mauch F, Luan S, Zou G, Whitham SA, Budworth PR, Tao Y, Xie Z, Chen X, Lam S, Kreps JA, Harper JF, Si-Ammour A, Mauch-Mani B, Heinlein M, Kobayashi K, Hohn T, Dangl JL, Wang X, Zhu T (2002) Expression profile matrix of Arabidopsis transcription factor genes suggests their putative functions in response to environmental stresses. Plant Cell **14:** 559-574.

Chen YF, Randlett MD, Findell JL, Schaller GE (2002) Localization of the ethylene receptor ETR1 to the endoplasmic reticulum of Arabidopsis. J Biol Chem **277:** 19861-19866.

Chilley PM, Casson SA, Tarkowski P, Hawkins N, Wang KL, Hussey PJ, Beale M, Ecker JR, Sandberg GK, Lindsey K (2006) The POLARIS peptide of Arabidopsis regulates auxin transport and root growth via effects on ethylene signaling. Plant Cell **18:** 3058-3072

Chilley PM, Casson SA, Tarkowski P, Hawkins N, Wang KLC, Hussey PJ, Beale M, Ecker JR, Sandberg GK, Lindsey K (2006) The POLARIS Peptide of Arabidopsis Regulates Auxin Transport and Root Growth via Effects on Ethylene Signaling. Plant Cell **18:** 3058-3072

Clarke JD, Volko SM, Ledford H, Ausubel FM, Dong X (2000) Roles of salicylic acid, jasmonic acid, and ethylene in cpr-induced resistance in arabidopsis. Plant Cell **12:** 2175-2190.

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J **16:** 735-743

Darwin C, Darwin F (1881) Darwins Gesammelte Werke Bd 13. Schweizerbart'sche Verlagsbuchhandlung, Stuttgart

De Paepe A, De Grauwe L, Bertrand S, Smalle J, Van der Straeten D (2005) The Arabidopsis mutant eer2 has enhanced ethylene responses in the light. J Exp Bot **56:** 2409-2420

Dellaert LMW (1980) Segregation frequencies of radiation-induced viable mutants in Arabidopsis thaliana (L.) Heynh. TAG Theoretical and Applied Genetics **57:** 137-143

Ecker JR (1995) The ethylene signal transduction pathway in plants. Science **268:** 667-675.

Ecker JR (2004) Reentry of the Ethylene MPK6 Module. Plant Cell **16:** 3169-3173

El Refy A, Perazza D, Zekraoui L, Valay JG, Bechtold N, Brown S, Hulskamp M, Herzog M, Bonneville JM (2003) The Arabidopsis KAKTUS gene encodes a HECT protein and controls the number of endoreduplication cycles. Mol Genet Genomics **270:** 403-414

Fischer U, Ikeda Y, Ljung K, Serralbo O, Singh M, Heidstra R, Palme K, Scheres B, Grebe M (2006) Vectorial information for Arabidopsis planar polarity is mediated by combined AUX1, EIN2, and GNOM activity. Curr Biol **16:** 2143-2149

Fluhr R, Mattoo AK (1996) Ethylene-biosynthesis and perception. Critical Reviews in Plant Sciences **15:** 479-523

Folkers U, Berger J, Hulskamp M (1997) Cell morphogenesis of trichomes in Arabidopsis: differential control of primary and secondary branching by branch initiation regulators and cell growth. Development **124:** 3779-3786

Fujimoto SY, Ohta M, Usui A, Shinshi H, Ohme-Takagi M (2000) Arabidopsis ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box-mediated gene expression. Plant Cell **12:** 393-404.

Furutani I, Watanabe Y, Prieto R, Masukawa M, Suzuki K, Naoi K, Thitamadee S, Shikanai T, Hashimoto T (2000) The SPIRAL genes are required for directional control of cell elongation in Aarabidopsis thaliana. Development **127:** 4443-4453

Gaertig J, Thatcher TH, Gu L, Gorovsky MA (1994) Electroporation-mediated replacement of a positively and negatively selectable beta-tubulin gene in Tetrahymena thermophila. Proc Natl Acad Sci U S A **91:** 4549-4553

Gao Z, Chen YF, Randlett MD, Zhao XC, Findell JL, Kieber JJ, Schaller GE (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

Garbers C, DeLong A, Deruere J, Bernasconi P, Soll D (1996) A mutation in protein phosphatase 2A regulatory subunit A affects auxin transport in Arabidopsis. EMBO J **15:** 2115-2124

Guo H, Ecker JR (2003) Plant responses to ethylene gas are mediated by SCF(EBF1/EBF2)-dependent proteolysis of EIN3 transcription factor. Cell **115:** 667-677

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

Hall AE, Bleecker AB (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 AE, Findell JL, Schaller GE, Sisler EC, Bleecker AB (2000) Ethylene perception by the ERS1 protein in Arabidopsis. Plant Physiol **123:** 1449-1458.

Hall MA, Connern CP, Harpham NV, Ishizawa K, Roveda-Hoyos G, Raskin I, Sanders IO, Smith AR, Turner R, Wood CK (1990) Ethylene: receptors and action. Symp Soc Exp Biol **44:** 87-110

Hamilton AJ, Bouzayen M, Grierson D (1991) Identification of a tomato gene for the ethylene-forming enzyme by expression in yeast. Proc Natl Acad Sci U S A **88:** 7434-7437

Hashimoto T (2002) Molecular genetic analysis of left-right handedness in plants. Philos Trans R Soc Lond B Biol Sci **357:** 799-808

Hazen SP, Borevitz JO, Harmon FG, Pruneda-Paz JL, Schultz TF, Yanovsky MJ, Liljegren SJ, Ecker JR, Kay SA (2005) Rapid array mapping of circadian clock and developmental mutations in Arabidopsis. Plant Physiol **138:** 990-997

Hirayama T, Kieber JJ, Hirayama N, Kogan M, Guzman P, Nourizadeh S, Alonso JM, Dailey WP, Dancis A, Ecker JR (1999) RESPONSIVE-TO-ANTAGONIST1, a Menkes/Wilson disease-related copper transporter, is required for ethylene signaling in Arabidopsis. Cell **97:** 383-393.

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

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

Hwang I, Chen HC, Sheen J (2002) Two-component signal transduction pathways in Arabidopsis. Plant Physiol **129:** 500-515.

Jenuwein T, Allis CD (2001) Translating the Histone Code. Science **293:** 1074-1080

Kieber JJ, Rothenberg M, Roman G, Feldmann KA, Ecker JR (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.

Klose RJ, Kallin EM, Zhang Y (2006) JmjC-domain-containing proteins and histone demethylation. Nat Rev Genet **7:** 715-727

Klose RJ, Yamane K, Bae Y, Zhang D, Erdjument-Bromage H, Tempst P, Wong J, Zhang Y (2006) The transcriptional repressor JHDM3A demethylates trimethyl histone H3 lysine 9 and lysine 36. Nature **442:** 312-316

Klose RJ, Yan Q, Tothova Z, Yamane K, Erdjument-Bromage H, Tempst P, Gilliland DG, Zhang Y, Kaelin JWG (2007) The Retinoblastoma Binding Protein RBP2 Is an H3K4 Demethylase. Cell **128:** 889

Konieczny A, Ausubel FM (1993) A procedure for mapping Arabidopsis mutations using co-dominant ecotype-specific PCR-based markers. Plant J **4:** 403-410

Kwak JM, Moon JH, Murata Y, Kuchitsu K, Leonhardt N, DeLong A, Schroeder JI (2002) Disruption of a Guard Cell-Expressed Protein Phosphatase 2A Regulatory Subunit, RCN1, Confers Abscisic Acid Insensitivity in Arabidopsis Article, publication date, and citation information can be found at www. plantcell. org/cgi/doi/10.1105/tpc. 003335. The Plant Cell Online **14:** 2849-2861

Larsen PB, Cancel JD (2003) Enhanced ethylene responsiveness in the Arabidopsis eer1 mutant results from a loss-of-function mutation in the protein phosphatase 2A A regulatory subunit, RCN1. Plant J **34:** 709-718

Larsen PB, Chang C (2001) The Arabidopsis eer1 Mutant Has Enhanced Ethylene Responses in the Hypocotyl and Stem. Plant Physiol **125:** 1061-1073.

Le J, Vandenbussche F, Van Der Straeten D, Verbelen J-P (2004) Position and cell type-dependent microtubule reorientation characterizes the early response of the Arabidopsis root epidermis to ethylene. Physiologia Plantarum **121:** 513-519

Lee N, Zhang J, Klose RJ, Erdjument-Bromage H, Tempst P, Jones RS, Zhang Y (2007) The trithorax-group protein Lid is a histone H3 trimethyl-Lys4 demethylase. Nat Struct Mol Biol

Lehman A, Black R, Ecker JR (1996) HOOKLESS1, an ethylene response gene, is required for differential cell elongation in the Arabidopsis hypocotyl. Cell **85:** 183-194.

Li H, Johnson P, Stepanova A, Alonso JM, Ecker JR (2004) Convergence of signaling pathways in the control of differential cell growth in Arabidopsis. Dev Cell **7:** 193-204

Li J, Dai X, Zhao Y (2006) A role for auxin response factor 19 in auxin and ethylene signaling in Arabidopsis. Plant Physiol **140:** 899-908

Li J, Yang H, Ann Peer W, Richter G, Blakeslee J, Bandyopadhyay A, Titapiwantakun B, Undurraga S, Khodakovskaya M, Richards EL, Krizek B, Murphy AS, Gilroy S, Gaxiola R (2005) Arabidopsis H+-PPase AVP1 Regulates Auxin-Mediated Organ Development. Science **310:** 121-125

Liang G, Klose RJ, Gardner KE, Zhang Y (2007) Yeast Jhd2p is a histone H3 Lys4 trimethyl demethylase. Nat Struct Mol Biol **14:** 243-245

Lim CJ, Yang KA, Hong JK, Choi JS, Yun DJ, Hong JC, Chung WS, Lee SY, Cho MJ, Lim CO (2006) Gene expression profiles during heat acclimation in Arabidopsis thaliana suspension-culture cells. J Plant Res **119:** 373-383

Lin WH, Ye R, Ma H, Xu ZH, Xue HW (2004) DNA chip-based expression profile analysis indicates involvement of the phosphatidylinositol signaling pathway in multiple plant responses to hormone and abiotic treatments. Cell Res **14:** 34

Liu Y, Zhang S (2004) Phosphorylation of 1-aminocyclopropane-1-carboxylic acid synthase by MPK6, a stress-responsive mitogen-activated protein kinase, induces ethylene biosynthesis in Arabidopsis. Plant Cell **16:** 3386-3399

Lorenzo O, Piqueras R, Sanchez-Serrano JJ, Solano R (2003) ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. Plant Cell **15:** 165-178.

Luschnig C, Gaxiola RA, Grisafi P, Fink GR (1998) EIR1, a root-specific protein involved in auxin transport, is required for gravitropism in Arabidopsis thaliana. Genes Dev **12:** 2175-2187

Marchant A, Kargul J, May ST, Muller P, Delbarre A, Perrot-Rechenmann C, Bennett MJ (1999) AUX1 regulates root gravitropism in Arabidopsis by facilitating auxin uptake within root apical tissues. Embo J **18:** 2066-2073

Martin C, Zhang Y (2005) The diverse functions of histone lysine methylation. Nat Rev Mol Cell Biol **6:** 838-849

Mathur J, Chua NH (2000) Microtubule stabilization leads to growth reorientation in Arabidopsis trichomes. Plant Cell **12:** 465-477

Mathur J, Spielhofer P, Kost B, Chua N (1999) The actin cytoskeleton is required to elaborate and maintain spatial patterning during trichome cell morphogenesis in Arabidopsis thaliana. Development **126:** 5559-5568

Meyer K, Leube MP, Grill E (1994) A protein phosphatase 2C involved in ABA signal transduction in Arabidopsis thaliana. Science **264:** 1452-1455

Mishina TE, Zeier J (2006) The Arabidopsis Flavin-Dependent Monooxygenase FMO1 Is an Essential Component of Biologically Induced Systemic Acquired Resistance. Plant Physiol. **141:** 1666-1675

Moussatche P, Klee HJ (2004) Autophosphorylation activity of the Arabidopsis ethylene receptor multigene family. J Biol Chem **279:** 48734-48741

Neljubow DN (1901) Über die horizontale Nutation der Stengel von Pisum sativum und einiger anderer Pflanzen. Beitrage und Botanik Zentralblatt **10:** 128-139

Noh B, Lee SH, Kim HJ, Yi G, Shin EA, Lee M, Jung KJ, Doyle MR, Amasino RM, Noh YS (2004) Divergent roles of a pair of homologous jumonji/zinc-finger-class transcription factor proteins in the regulation of Arabidopsis flowering time. Plant Cell **16:** 2601-2613

O'Donovan MC, Oefner PJ, Roberts SC, Austin J, Hoogendoorn B, Guy C, Speight G, Upadhyaya M, Sommer SS, McGuffin P (1998) Blind analysis of denaturing high-performance liquid chromatography as a tool for mutation detection. Genomics **52:** 44-49

O'Malley RC, Rodriguez FI, Esch JJ, Binder BM, O'Donnell P, Klee HJ, Bleecker AB (2005) Ethylene-binding activity, gene expression levels, and receptor system output for ethylene receptor family members from Arabidopsis and tomato. Plant J **41:** 651-659

Ohshima K, Taniyama T, Yamanaka T, Ishikawa M, Naito S (1998) Isolation of a Mutant ofArabidopsis thalianaCarrying Two Simultaneous Mutations Affecting Tobacco Mosaic Virus Multiplication within a Single Cell. Virology **243:** 472

Okada K, Shimura Y (1990) Reversible Root-Tip Rotation In Arabidopsis Seedlings Induced By Obstacle-Touching Stimulus. Science **250:** 274-276

Olmedo G, Guo H, Gregory BD, Nourizadeh SD, Aguilar-Henonin L, Li H, An F, Guzman P, Ecker JR (2006) ETHYLENE-INSENSITIVE5 encodes a 5'-->3' exoribonuclease required for regulation of the EIN3-targeting F-box proteins EBF1/2. Proc Natl Acad Sci U S A **103:** 13286-13293

Perazza D, Herzog M, Hulskamp M, Brown S, Dorne A-M, Bonneville J-M (1999) Trichome Cell Growth in Arabidopsis thaliana Can Be Derepressed by Mutations in at Least Five Genes. Genetics **152:** 461-476

Petruzzelli L, Coraggio I, Leubner-Metzger G (2000) Ethylene promotes ethylene biosynthesis during pea seed germination by positive feedback regulation of 1-aminocyclo-propane-1-carboxylic acid oxidase. Planta **211:** 144-149

Potuschak T, Lechner E, Parmentier Y, Yanagisawa S, Grava S, Koncz C, Genschik P (2003) EIN3-dependent regulation of plant ethylene hormone signaling by two arabidopsis F box proteins: EBF1 and EBF2. Cell **115:** 679-689

Potuschak T, Vansiri A, Binder BM, Lechner E, Vierstra RD, Genschik P (2006) The exoribonuclease XRN4 is a component of the ethylene response pathway in Arabidopsis. Plant Cell **18:** 3047-3057

Qu X, Hall BP, Gao Z, Schaller GE (2007) A strong constitutive ethylene-response phenotype conferred on Arabidopsis plants containing null mutations in the ethylene receptors ETR1 and ERS1. BMC Plant Biol **7:** 3

Rashotte AM, DeLong A, Muday GK (2001) Genetic and chemical reductions in protein phosphatase activity alter auxin transport, gravity response, and lateral root growth. Plant Cell **13:** 1683-1697

Resnick JS, Wen CK, Shockey JA, Chang C (2006) REVERSION-TO-ETHYLENE SENSITIVITY1, a conserved gene that regulates ethylene receptor function in Arabidopsis. Proc Natl Acad Sci U S A **103:** 7917-7922

Riechmann JL, Meyerowitz EM (1998) The AP2/EREBP family of plant transcription factors. Biol Chem **379:** 633-646.

Rodriguez FI, Esch JJ, Hall AE, Binder BM, Schaller GE, Bleecker AB (1999) A copper cofactor for the ethylene receptor ETR1 from Arabidopsis. Science **283:** 996-998.

Roman G, Ecker JR (1995) Genetic analysis of a seedling stress response to ethylene in Arabidopsis. Philos Trans R Soc Lond B Biol Sci **350:** 75-81.

Roman G, Lubarsky B, Kieber JJ, Rothenberg M, Ecker JR (1995) Genetic analysis of ethylene signal transduction in Arabidopsis thaliana: five novel

mutant loci integrated into a stress response pathway. Genetics **139:** 1393-1409.

Schaller GE, Bleecker AB (1995) Ethylene-binding sites generated in yeast expressing the Arabidopsis ETR1 gene. Science **270:** 1809-1811.

Schaller GE, Kieber JJ (2002) Ethylene. American Society of Plant Biologists, Rockville

Schwab B, Folkers U, Ilgenfritz H, Hulskamp M (2000) Trichome morphogenesis in Arabidopsis. Philos Trans R Soc Lond B Biol Sci **355:** 879-883

Sedbrook JC, Ehrhardt DW, Fisher SE, Scheible WR, Somerville CR (2004) The Arabidopsis sku6/spiral1 gene encodes a plus end-localized microtubule-interacting protein involved in directional cell expansion. Plant Cell **16:** 1506-1520

Seward DJ, Cubberley G, Kim S, Schonewald M, Zhang L, Tripet B, Bentley DL (2007) Demethylation of trimethylated histone H3 Lys4 in vivo by JARID1 JmjC proteins. Nat Struct Mol Biol **14:** 240

Shi Y, Lan F, Matson C, Mulligan P, Whetstine JR, Cole PA, Casero RA, Shi Y (2004) Histone Demethylation Mediated by the Nuclear Amine Oxidase Homolog LSD1. Cell **119:** 941

Shibaoka H (1994) Plant Hormone-Induced Changes in the Orientation of Cortical Microtubules: Alterations in the Cross-linking Between Microtubules and the Plasma Membrane. Annual Review of Plant Physiology and Plant Molecular Biology **45:** 527-544

Shirley BW, Hanley S, Goodman HM (1992) Effects of Ionizing Radiation on a Plant Genome: Analysis of Two Arabidopsis transparent testa Mutations. The Plant Cell Online **4:** 333-347

Sisler EC (1991) Ethylene-binding components in plants. CRC Press, Bocca Raton

Sistrunk ML, Antosiewicz DM, Purugganan MM, Braam J (1994) Arabidopsis TCH3 encodes a novel Ca2+ binding protein and shows environmentally induced and tissue-specific regulation. Plant Cell **6:** 1553-1565.

Smalle J, Haegman M, Kurepa J, Van Montagu M, Straeten DV (1997) Ethylene can stimulate Arabidopsis hypocotyl elongation in the light. Proc Natl Acad Sci U S A **94:** 2756-2761.

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

Souret FF, Kastenmayer JP, Green PJ (2004) AtXRN4 degrades mRNA in Arabidopsis and its substrates include selected miRNA targets. Mol Cell **15:** 173-183

Stepanova AN, Ecker JR (2000) Ethylene signaling: from mutants to molecules. Curr Opin Plant Biol **3:** 353-360.

Stepanova AN, Hoyt JM, Hamilton AA, Alonso JM (2005) A Link between ethylene and auxin uncovered by the characterization of two root-specific ethylene-insensitive mutants in Arabidopsis. Plant Cell **17:** 2230-2242

Sugimoto K, Himmelspach R, Williamson RE, Wasteneys GO (2003) Mutation or Drug-Dependent Microtubule Disruption Causes Radial Swelling without Altering Parallel Cellulose Microfibril Deposition in Arabidopsis Root Cells. Plant Cell **15:** 1414-1429

Swarup R, Kramer EM, Perry P, Knox K, Leyser HMO, Haseloff J, Beemster GTS, Bhalerao R, Bennett MJ (2005) Root gravitropism requires lateral root cap and epidermal cells for transport and response to a mobile auxin signal. Nat Cell Biol **7:** 1057

Szymanski DB, Lloyd AM, Marks MD (2000) Progress in the molecular genetic analysis of trichome initiation and morphogenesis in Arabidopsis. Trends Plant Sci **5:** 214-219

Tatsuki M, 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

Telfer A, Bollman KM, Poethig RS (1997) Phase change and the regulation of trichome distribution in Arabidopsis thaliana. Development **124:** 645-654

Theologis A (1989) Auxin-regulated gene expression in plants. Biotechnology **12:** 229-243

Thitamadee S, Tuchihara K, Hashimoto T (2002) Microtubule basis for left-handed helical growth in Arabidopsis. Nature **417:** 193-196

Tsuchisaka A, Theologis A (2004) Heterodimeric interactions among the 1-amino-cyclopropane-1-carboxylate synthase polypeptides encoded by the Arabidopsis gene family. Proc Natl Acad Sci U S A **101:** 2275-2280

Tsuchisaka A, Theologis A (2004) Unique and overlapping expression patterns among the Arabidopsis 1-amino-cyclopropane-1-carboxylate synthase gene family members. Plant Physiol **136:** 2982-3000

Uchida A, Yamamoto KT (2002) Effects of mechanical vibration on seed germination of Arabidopsis thaliana (L.) Heynh. Plant Cell Physiol **43:** 647-651.

Vandenbussche F, Smalle J, Le J, Saibo NJ, De Paepe A, Chaerle L, Tietz O, Smets R, Laarhoven LJ, Harren FJ, Van Onckelen H, Palme K, Verbelen JP, Van Der Straeten D (2003) The Arabidopsis Mutant alh1 Illustrates a Cross Talk between Ethylene and Auxin. Plant Physiol **131:** 1228-1238.

Vogel JP, Woeste KE, Theologis A, Kieber JJ (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.

Wang H, Lee MM, Schiefelbein JW (2002) Regulation of the cell expansion gene RHD3 during Arabidopsis development. Plant Physiol **129:** 638-649.

Wang KL, Li H, Ecker JR (2002) Ethylene biosynthesis and signaling networks. Plant Cell **14 Suppl:** S131-151

Wang KL, Yoshida H, Lurin C, Ecker JR (2004) Regulation of ethylene gas biosynthesis by the Arabidopsis ETO1 protein. Nature **428:** 945-950

Wang W, Hall AE, O'Malley R, Bleecker AB (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.

Weigel D, Ahn JH, Blazquez MA, Borevitz JO, Christensen SK, Fankhauser C, Ferrandiz C, Kardailsky I, Malancharuvil EJ, Neff MM, Nguyen JT, Sato S, Wang Z-Y, Xia Y, Dixon RA, Harrison MJ, Lamb CJ, Yanofsky MF, Chory J (2000) Activation Tagging in Arabidopsis. Plant Physiol. **122:** 1003-1014

Whittaker DJ, Smith GS, Gardner RC (1997) Expression of ethylene biosynthetic genes in Actinidia chinensis fruit. Plant Mol Biol **34:** 45-55

Woeste KE, Kieber JJ (2000) A strong loss-of-function mutation in RAN1 results in constitutive activation of the ethylene response pathway as well as a rosette-lethal phenotype. Plant Cell **12:** 443-455.

Woeste KE, Ye C, Kieber JJ (1999) Two Arabidopsis mutants that overproduce ethylene are affected in the posttranscriptional regulation of 1-aminocyclopropane-1-carboxylic acid synthase. Plant Physiol **119:** 521-530.

Xiong L, Gong Z, Rock CD, Subramanian S, Guo Y, Xu W, Galbraith D, Zhu JK (2001) Modulation of abscisic acid signal transduction and biosynthesis by an Sm-like protein in Arabidopsis. Dev Cell **1:** 771-781

Yang S, Hofmann N (1984) Ethylene Biosynthesis and its Regulation in Higher-Plants. Annual Review of Plant Physiology and Plant Molecular Biology **35:** 155-189

Yoshida H, Wang KL, Chang CM, Mori K, Uchida E, Ecker JR (2006) The ACC synthase TOE sequence is required for interaction with ETO1 family proteins and destabilization of target proteins. Plant Mol Biol **62:** 427-437

Zhao XC, Qu X, Mathews DE, Schaller GE (2002) Effect of ethylene pathway mutations upon expression of the ethylene receptor ETR1 from Arabidopsis. Plant Physiol **130:** 1983-1991.

Zhao Y, Christensen SK, Fankhauser C, Cashman JR, Cohen JD, Weigel D, Chory J (2001) A Role for Flavin Monooxygenase-Like Enzymes in Auxin Biosynthesis. Science **291:** 306-309