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Dissecting the ethylene
pathway of Arabidopsis
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Abstract
The plant hormone ethylene regulates growth, development and stress responses. In recent
times, various genomic and proteomic approaches have been initiated to understand both the
range of ethylene responses in the plant and the mechanism of signal transduction.

Transcriptional profiling experiments reveal broad-ranging effects of ethylene upon gene
regulation, with up to 7 per cent of the genes examined demonstrating a significant level of
response in one study. Both transcriptional and post-transcriptional mechanisms regulate the
expression of components within the ethylene signal transduction pathway. The importance of
post-transcriptional regulation via the ubiquitin/proteasome-mediated degradation pathway is
apparent in studies on the accumulation of ethylene insensitive 3 (EIN3), a key transcription
factor in the pathway. Protein complexes also play a role in modulating ethylene signal
transduction, with interactions between the ethylene receptors and the Raf-like kinase
constitutive triple response-1 (CTR1) being required for ethylene perception at the
endoplasmic reticulum. In this paper, recent developments in unravelling the transcriptional
and post-transcriptional regulation of the ethylene signalling and response pathways are
considered, along with the latest developments in unravelling the biochemical mechanism
behind ethylene perception.

INTRODUCTION
As with other multicellular eukaryotes,
hormones are a driving force behind
many physiological and developmental
processes in plants. The sessile nature of
the plant, however, produces unique
challenges that animals do not have to
face. A plant must survive where it
germimates, contending with pathogen
attacks, nutrient availability and
environmental variations (eg light,
temperature, water accessibility). To
confront these challenges, plants employ
complex signalling networks to detect and
respond to external cues from the
environment in conjunction with internal
developmental and physiological cues.

Plant hormones play essential roles in
coordinating external and internal signals
to elicit the appropriate growth and
developmental responses, often in
concert, as a means precisely to regulate
responses both temporally and spatially.

One of the simplest plant hormones is
the gaseous molecule ethylene. Ethylene
is best known for its role in the regulation
of fruit ripening. Indeed, ethylene has
been used for thousands of years by
farmers, often unwittingly, to induce
ripening.1 It was not until the 20th
century, however, that ethylene was
recognised as a phytohormone when it
was shown to be a plant-produced
molecule that induced various
physiological effects.2–4 Ethylene is now
known to regulate many developmental
processes throughout the plant’s life cycle,
from germination to senescence, and is
also involved in responses to
environmental stimuli such as stress and
pathogen attack. Many biotechnological
applications target ethylene’s role in
promoting senescence, fruit ripening and
abscission. Ethylene also controls aspects
of flower development, the release of
dormancy and various defence
responses.4–6 At the cellular level,
ethylene controls cell elongation, thereby
regulating root growth, stem (or
hypocotyl) growth and apical hook
Ethylene signal transduction makes use of positive and negative regulators.

Figure 1: Ethylene signal transduction. In the absence of ethylene, constitutive triple response 1 (CTR1) is maintained in an active state by the receptors, which serves to inhibit downstream components and thus the ethylene response. In addition, the transcription factor ethylene insensitive 3 (EIN3) is constantly degraded through the action of EIN3 binding F-box (EBF1) and EBF2 via the proteasome-mediated degradation pathway. Upon binding of ethylene, the receptor inactivates CTR1. This relieves the repression on downstream signalling components, thus allowing for activation of the EIN3/EIL transcription factors and ethylene responses. Ethylene promotes accumulation of EIN3 by repressing the action of EBF1 and EBF2. The subcellular location of components is shown, where known. Ovals represent the active conformations of proteins; rectangles represent inactive conformations of proteins. Cu, copper; ER, endoplasmic reticulum; ERF, ethylene-responsive element binding factor; ETR, ethylene resistant; ERS, ethylene response sensor; MAPKKK, mitogen-activated protein kinase kinase kinase.

Etheridge et al. components of the ethylene signalling pathway in the genetic model plant Arabidopsis thaliana.

The ethylene signal transduction pathway deduced from genetic analyses can be modelled as a linear pathway from perception to transcriptional regulation (Figure 1). A significant feature of the ethylene signalling pathway is that it incorporates both positive and negative regulators. Ethylene is perceived by a family of receptors that have similarity to histidine kinases, an evolutionarily ancient class of signalling proteins originally identified in bacteria. The ethylene receptors interact with and apparently regulate the activity of constitutive triple response 1 (CTR1), a Ser/Thr kinase that is a negative regulator of ethylene signalling and which actively suppresses the ethylene responses in the absence of ethylene.7 CTR1 has sequence similarity to Raf, a mitogen-activated protein kinase kinase (MAPKKK);7 consequently, a MAP kinase cascade has been postulated to act downstream of CTR1, although the components of this cascade have been the subject of recent debate.8–10 When ethylene binds to the receptors, CTR1 is thought to be inactivated. This relieves the suppression on downstream signalling elements, resulting in the activation of ethylene insensitive 2 (EIN2), a membrane-bound protein with similarity to Nramp metal-ion transporters,11 and the activation of downstream transcriptional regulators.12,13 The most significant family of transcription factors is composed of EIN3 and the EIN3-like (EIL) proteins, of which EIN3 appears to play the predominant role in regulating the ethylene response.13–15 The EIN3/EIL family function in a transcriptional cascade that regulates expression of a variety of genes, including ethylene-responsive factor 1 (ERF1),12,13 one of the ethylene-responsive element binding factor (ERF) family of transcription factors.16

Kinetic analysis indicates that ethylene’s effect on seedling growth is exerted in two phases. The first phase is a rapid but transient response that occurs within 15
The response of seedlings to ethylene is biphasic

The first phase is very sensitive to low ethylene levels but is not dose dependent, nor does it require the function of the transcription factors EIN3 and EIL1. This suggests that the initial phase may be post-transcriptionally regulated to allow for a rapid response to small changes in ethylene concentration, although further research is necessary to confirm this hypothesis. The first phase is followed by a sustained and slower response that is both dose dependent and EIN3/EIL1 dependent, consistent with a transcriptional requirement for this phase of the growth response.

The full scope of transcriptional and post-transcriptional regulatory processes on ethylene signalling is only now being appreciated, in part by taking advantage of mutants in the ethylene signal transduction pathway. These mutants have been of great importance in dissecting ethylene's role in transcriptional regulation via microarray experiments, and also in revealing the role of post-transcriptional regulation of the ethylene response. Proteomic analyses using these mutants have revealed that a receptor complex is responsible for perceiving and transducing the ethylene signal. Recent developments in these topics will be discussed in the following sections, to highlight the role that functional genomics and proteomics is playing in exploring the ethylene signalling pathway.

Ethylene transcriptionally regulates genes involved in a wide range of biological processes

Transcriptional profiling has been used by several groups to uncover ethylene-regulated genes. One of the first groups to look at ethylene regulation used a multipronged approach to determine the genes involved in the defence response of Arabidopsis. Although only a small proportion of the genome was analysed on these microarrays (2,375 expressed sequence tags [ESTs]), the analysis identified genes that were coordinately regulated by multiple defence elicitors such as salicylic acid, methyl jasmonate and ethylene. This result highlights the cross-talk that occurs between signalling molecules to precisely regulate the defence response.

More recently, in an experiment using a 22,000-gene Affymetrix DNA microarray, the effects of ethylene on transcription were analysed in dark-grown seedlings that had received constant exposure to ethylene. Approximately 2.9 per cent of the genes tested showed a significant (greater than twofold) change in transcription, the majority of which were repressed. Genes involved in many different biological processes, from metabolism to signal transduction, were regulated by ethylene. Several genes containing AP2 domains, including ERF1, were identified as being ethylene induced, four of which contained known plant-specific DNA-binding domains. Insertional mutants for each of these four genes were identified and, although single knockout mutants did not demonstrate an altered ethylene response, double mutants were partially ethylene insensitive, thus confirming a role for these genes in mediating the ethylene response.

In an EST-based microarray study representing 5,955 genes, ethylene’s effect on expression in the leaves of older plants was examined and was also compared with the expression profiles of several known mutants in the ethylene signalling pathway. The dominant mutation etr1-1 renders ethylene resistant 1 (ETR1) incapable of binding ethylene, and results in a plant that is ethylene insensitive. By contrast, the loss-of-function mutation ctr1-1 results in a constitutive ethylene response. This study focused on genes whose expression is affected by long-term ethylene treatment by examining the aerial parts of adult (24-day-old) plants after treatment with ethylene for 24 hours. The changes in gene expression between the two mutants were examined and compared with wild-type plants treated with ethylene. A higher percentage of ethylene-regulated genes were identified (7 per cent) in this study than in that of Alonso et al., but this may reflect the...
smaller number of genes represented on the array and/or the differences in ethylene treatment conditions. As was found in the study by Alonso et al.,\textsuperscript{20} ethylene-regulated genes were implicated in many different biological processes, including ethylene biosynthesis and perception, other hormone responses, stress responses and metabolism.\textsuperscript{21} Surprisingly, comparison of genes regulated in the constitutive ethylene response mutant \textit{ctr1-1} with those in wild-type plants treated with ethylene for 24 hours revealed a significant number of genes that were either differentially or oppositely regulated in these sample types.\textsuperscript{21} These data are indicative of the dose-dependent nature of the ethylene response and also suggest that a negative feedback loop may be in effect.\textsuperscript{21}

Using a similar mutant versus wild-type approach, another group analysed an EST-based microarray representing 6,008 genes in parallel with the polymerase chain reaction-based approach of cDNA-amplified fragment length polymorphism (cDNA-AFLP).\textsuperscript{23} In this case, a different ethylene-insensitive mutant (\textit{ein2-1}) was used along with the constitutive ethylene response mutant \textit{ctr1-1}. Loss-of-function mutations in \textit{EIN2} result in the strongest ethylene insensitivity of all the ethylene insensitive mutations identified in \textit{Arabidopsis},\textsuperscript{14} and so comparing this genotype to the constitutive ethylene response mutant \textit{ctr1-1} is likely to yield the greatest difference in expression of ethylene-regulated genes.\textsuperscript{23} In this study, the transcriptional changes in adult (19-day-old) plants was examined after short- (ten-minute) and medium- (6-hour) term ethylene exposure. To avoid complications in gene expression due to circadian rhythms, the authors also discarded genes that were differentially regulated over time in the hope of obtaining a more accurate representation of ethylene-regulated genes.\textsuperscript{23} A total of 214 genes (3.6 per cent) were found to be ethylene regulated and those genes were placed into distinct clusters of expression patterns.\textsuperscript{23} The largest clusters were those that were either repressed or induced by ethylene, but there were several smaller clusters with genes that were distinctly regulated by ethylene after short- or long-term exposure. These experiments confirm that ethylene-regulated transcriptional activation is dose dependent and that short-term ethylene treatments can result in significant transcriptional changes.\textsuperscript{23} As in the other microarray experiments, genes were identified that are involved in many different biological processes. Interestingly, the cluster of repressed genes primarily encoded proteins involved in metabolism, defence and transport. This study also revealed that several genes involved in ubiquitin/26S proteasome-mediated degradation are ethylene regulated, especially during the early responses to ethylene.\textsuperscript{23}

These experiments have revealed details on the global transcriptional changes that take place in response to ethylene, lending insight into the time-dependent nature of the transcriptional response to ethylene and into how mutations in signalling pathway components affect this response. The differences in the number of ethylene-regulated genes uncovered by each of these microarray analyses probably reflect the diverse experimental approaches taken by the researchers. The insights into the global expression patterns of ethylene regulation will be the foundation for future investigations to ascertain ethylene’s roles in the many biological processes under its control.

\textbf{POST-TRANSCRIPTIONAL REGULATION}

Precise control of the ethylene response is achieved through complex regulation of the ethylene biosynthesis and signalling pathways. Ethylene has been shown to regulate the transcription of components, such as certain biosynthesis and receptor genes, involved in signalling; however, recent studies have shown that post-transcriptional regulation also plays an important role. One of the post-
transcriptional mechanisms used in the regulation of ethylene signalling involves ubiquitin/26S proteasome-mediated degradation, a process that has been shown to regulate various physiological processes, including growth and development, hormone responses and defence responses. Three enzyme complexes, the ubiquitin-activating (E1) enzyme, the ubiquitin-conjugating (E2) enzyme and the ubiquitin-ligating enzyme (E3 ligase), work together to covalently attach ubiquitin moieties to target proteins destined for degradation. E3 ligase confers specificity to the reaction by recognition of degradation targets and by mediating interaction with the E2 enzyme to attach multiple ubiquitin moieties. The polyubiquitinated protein is then recognised and degraded by the 26S proteasome. Within *Arabidopsis*, there are more than 1,000 potential E3 ligases, each with distinct target specificities, indicating that the plant has a substantial capacity for post-transcriptional regulation through proteasome-mediated degradation. 

Proteasome-mediated degradation is thought to regulate ethylene biosynthesis through one of the key enzymes involved in ethylene biosynthesis 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS). At least one ACS isoform, ACS4, is targeted to the proteasome-mediated degradation pathway by an N-terminal signal. Protein levels of another ACS isoform, ACS5, are negatively regulated through interaction with ETO1, an E3-ligase component. Phosphorylation may be involved in ACS turnover, as has been seen for proteasome-mediated degradation in animal systems. Recent data suggest that another ACS isoform, ACS6, is phosphorylated by a MAP kinase (MPK6), and that this phosphorylation results in ACS6 accumulation and a concomitant increase in ethylene production. In this case, phosphorylation protects ACS6 from degradation, potentially through a proteasome-mediated pathway. The next downstream component in ethylene biosynthesis, ACC oxidase (ACO), also appears to be regulated by proteasome-mediated degradation. Levels of two components involved in activating the E3 ligase, related to ubiquitin (RUB) and RUB-conjugating enzyme (RCE), appear to affect ACO levels and thus activity. Considering that there are multiple isoforms of both ACS and ACO, further analysis should reveal whether all members of this family are regulated by proteasome-mediated degradation or whether this regulation is specific for certain isoforms.

There are several points of post-transcriptional regulation that directly affect ethylene signal transduction. Some mutations in the ethylene-binding domain of ETR1 result in increased protein levels not reflected in changes in mRNA levels, suggesting that at least one of the ethylene receptors is post-transcriptionally regulated. Perturbation of ethylene binding with silver causes a similar effect, thus suggesting that ligand binding may regulate receptor turnover. This type of ligand-mediated receptor turnover is common in animal systems, but further analysis of ethylene receptor turnover is required to confirm this proposed mechanism in plants.

The activity of a key transcription factor responsible for the ethylene response, EIN3, is also controlled by proteasome-mediated degradation. In the absence of ethylene, EIN3 is continuously degraded, thus preventing activation of its transcriptional targets (Figure 1). EIN3 protein levels increase in the presence of ethylene, thus enabling this transcription factor to activate its targets and initiate the ethylene response. Turnover of EIN3 is regulated by two E3 ligases, EIN3 binding F-box (EBF) 1 and EBF2, which mediate ubiquitination of EIN3 and thus promote its degradation. Mutant *Arabidopsis* plants that are deficient in either or both of these E3 ligases show increased EIN3 levels. Although EBF1 and EBF2 function in concert to regulate EIN3 levels,
differences in their regulation suggest subtly different roles. *EBF1* is not transcriptionally regulated by ethylene; however, its basal levels in the plant are higher than those of *EBF2*, suggesting that *EBF1* is involved in the initial ethylene response. *EBF1* may regulate *EIN3* levels in a dose-dependent manner, stimulating turnover at low ethylene concentrations until a certain ethylene threshold has been reached. By contrast, *EBF2* transcription is induced by ethylene, thus *EBF2* may induce degradation of *EIN3* at elevated ethylene concentrations. This would facilitate a rapid response to changes in ethylene levels because, upon removal of ethylene, *EIN3* would be degraded by *EBF2* and thus no longer induce the ethylene response. As previously described, the kinetics of the ethylene response is biphasic, available evidence suggesting that the first phase is post-transcriptionally regulated and responsive to low levels of ethylene. These characteristics are consistent with *EBF1*-mediated regulation of *EIN3* protein levels, thereby raising the possibility that this initial phase in the ethylene response is regulated by *EBF1*.

The specific mechanism behind ethylene-mediated turnover of *EIN3* through *EBF1* and *EBF2* has yet to be resolved, although several models have been proposed. These models differ in the proposed targets of ethylene’s regulatory action. In one model, ethylene is proposed to affect stability, activity or localisation of *EBF1* and *EBF2*, thus resulting in effects on *EIN3* accumulation. Alternatively, ethylene could induce a post-transcriptional modification of *EIN3*, such as phosphorylation, which may affect the ability of *EIN3* to interact with *EBF1* or *EBF2* and thus enhance the stabilisation of *EIN3*. Whichever the case, these studies illuminate the complexity of *EIN3* regulation and support the theory that this protein is a major control point for the ethylene signalling pathway.

**The Ethylene Receptor Complex**

Although hormone receptors are typically localised to the plasma membrane, the ethylene receptor *ETR1* is localised to the endoplasmic reticulum (ER) membrane. This does not pose a problem for ethylene perception because ethylene can readily diffuse through both aqueous and lipid environments. There are several potential advantages for a receptor localised to the ER, which may have relevance to ethylene signal transduction. For example, the ER is the site of such cellular functions as calcium storage, protein synthesis, lipid metabolism and defence responses. The ER is also connected to other organelles through its endomembrane network, and could thus provide the receptors with ready access to processes throughout the cell, potentially facilitating communication between organelles and other signalling pathways.

*CTR1* has also been demonstrated to localise to the ER membrane, even though it is not predicted to contain transmembrane domains. This membrane association is apparently due to a physical interaction between *CTR1* and the ethylene receptors based on *in vivo* and *in vitro* experiments. It has also been found that ethylene treatment increases the amount of *CTR1* protein associated with the ER membrane in a post-transcriptional manner, possibly through stabilisation of the protein by association with the receptors. The association between *CTR1* and the receptors is required for proper functioning of *CTR1* based on mutational analysis. The *ctr1-8* mutation was originally isolated in a screen for plants showing a constitutive ethylene response phenotype, indicating that *CTR1* was not functional in these plants. The *ctr1-8* mutation results in a single amino acid change in the N-terminal half of *CTR1*, in a region distinct from the C-terminal kinase domain. Based on two-hybrid analysis, this mutation abolishes the ability of...
CTR1 to interact with the ethylene receptor ETR1. In addition, in plants with the ctr1-8 mutation, the mutant CTR1 protein is to be no longer found associated with membranes but instead is found in the soluble fraction, apparently due to its inability to associate with the receptors. Thus, proper functioning of CTR1, in addition to requiring its kinase activity, also requires a physical association with the ethylene receptors to form a protein complex.

The mechanism behind the activation of CTR1 by the receptors is not clear. CTR1 is similar to the protein kinase Raf, and thus may have similar characteristics — such as a domain that autoinhibits its kinase activity. In this model, interaction with the receptor maintains CTR1 in a kinase-active conformation. Upon ethylene binding, the receptor induces a conformational change in CTR1 which allows the autoinhibitory domain to inhibit kinase activity. Another possibility is that the receptors regulate CTR1 activity through phosphorylation, a common means of regulating enzymatic activity. Two ethylene receptors have histidine kinase activity and the others may have Ser/Thr kinase activity. Further analysis should help to elucidate the mechanism behind the regulation of CTR1 activity. Whichever mechanism is shown to be correct, current evidence indicates that the ethylene receptors and CTR1 function together as a protein complex, and that formation of this complex is essential to ethylene signal transduction.

LOOKING AHEAD

The isolation of Arabidopsis mutants with altered ethylene responses was pivotal in the identification of components comprising the ethylene signal transduction pathway. These mutants have played important roles in the analysis and testing of models for ethylene signalling. In this paper, the application of these mutants to microarray analysis and the characterisation of the ethylene receptor/CTR1 complex have been made apparent. Analysis of how these mutants are affected at the genomic and proteomic levels has barely been initiated and, in the future, will lead to new insights into the mechanism of ethylene signalling and responses.

The microarray data gathered to date have been based on the analysis of whole plants or aerial tissues, an approach that averages out changes in gene expression across multiple tissues and, as a result, masks subtle and/or localised effects. Given the range of processes in which ethylene plays a role, it is clear that more focused approaches are required. Greater focus will resolve changes in gene expression for individual tissues and in specific cell types, and will thus result in a clearer picture of the diverse roles that ethylene plays in plant growth and development. Such focused expression pattern profiling has been performed to good effect in the analysis of differing gene expression in specific root cell types and points to the fascinating direction that future investigations of this type will take.

What is also becoming apparent, even from the limited analyses performed to date, is the importance of post-transcriptional regulation in the modulation of the ethylene response. Many of the proteomics strategies have only begun to be applied to the study of ethylene signalling. For example, an investigation of ethylene-regulated proteins by two-dimensional gel electrophoresis revealed that two proteins, a glutathione S-transferase and a pyrophosphatase-like protein, are significantly induced by ethylene. More complete analyses are likely to reveal a greater abundance of ethylene-regulated proteins and, with coordinate microarray data, will yield an improved understanding of the role of post-transcriptional regulation in ethylene signal transduction.

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