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Rapid Kinetic Analysis of Ethylene Growth Responses in Seedlings: New Insights into Ethylene Signal Transduction

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Abstract

Ethylene is a phytohormone that influences diverse processes in plants. Ethylene causes various changes in etiolated seedlings that differ between species and include reduced growth of shoots and roots, increased diameter of shoots, agravitropic growth, initiation of root hairs, and increased curvature of the apical hook. The inhibition of growth in etiolated seedlings has become widely used to screen for and identify mutants. This approach has led to an increased understanding of ethylene signaling. Most studies use end-point analysis after several days of exposure to assess the effects of ethylene. Recently, the use of time-lapse imaging has re-emerged as an experimental method to study the rapid kinetics of ethylene responses. This review outlines the historical use of ethylene growth kinetic studies and summarizes the recent use of this approach coupled with molecular biology to provide new insights into ethylene signaling.

Key words: Ethylene; Growth kinetics; Signal transduction; Time-lapse imaging; Receptors

Introduction

Ethylene is a simple, unsaturated hydrocarbon. Despite its chemical simplicity, ethylene affects many diverse processes throughout the lifetime of a plant, including seed germination, growth, formation of the apical hook, organ senescence, fruit ripening, abscission, gravitropism, and responses to various stresses (Mattoo and Suttle 1991; Abeles and others 1992).

Of these myriad processes, much attention has focused on the effects of ethylene on etiolated seedlings. Neljubov (1901) is credited with the discovery that ethylene is biologically active. He showed that ethylene was the active compound in illuminating gas that caused horizontal growth of etiolated pea seedlings. Later work demonstrated that ethylene causes a number of changes in etiolated seedlings that vary from species to species and include reduced growth of the hypocotyl and root, increased radial expansion of the hypocotyl, altered geotropism, and increased tightening of the apical hook (Abeles and others 1992). The growth inhibition response from prolonged exposure to ethylene has proven to be a sensitive and easily quantified

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bioassay that has been used to screen for mutants to gain information about the ethylene signaling pathway (Bleecker and others 1988; Guzmán and Ecker 1990). This bioassay has also been used to characterize the relationship between ethylene dose and physiological response, providing information of rate-limiting steps in the signal transduction pathway (Goeschl and Kays 1975; Chen and Bleecker 1995). Although this end-point analysis of seedling growth continues to be an informative bioassay, it has the limitation of only examining the long-term, end-point effects of ethylene on seedlings. This means transient events will be overlooked. Seedlings are dynamic in their growth, constantly integrating hormonal, developmental, and environmental signals. Trying to analyze this solely with end-point analysis is likely to miss subtle changes.

Heisenberg (1958) wrote regarding subatomic events that ''... since the measuring device has been constructed by the observer ... we have to remember that what we observe is not nature in itself but nature exposed to our method of questioning.'' This idea also applies to biology, where our understanding is often limited by the measuring devices and methods available. This review focuses on a re-emerging method to examine the rapid time course of the effects of ethylene on intact, growing seedlings. Combining this kinetic approach with mutational analysis has recently provided new insights into various aspects of ethylene signaling that would have remained obscure using end-point analysis of seedling growth.

Early Kinetic Studies of Growth Regulation by Ethylene

The growth and movement of seedlings have been subjects of study for over 125 years (Darwin and Darwin 1880 and references therein). It was over 70 years ago that van der Laan used manual, time-lapse photographic techniques to examine the kinetics of growth inhibition by ethylene on intact, etiolated pea and oat seedlings. It was found in this study that growth inhibition could occur with a delay as short as 30 min (van der Laan 1934). Similar results were obtained by Michener (1938) for pea by measuring the heights of seedlings at various times after applying ethylene.

These results were generally confirmed and expanded in the 1970s and 1980s by several research groups using a variety of methodologies capable of measuring the length of intact, growing roots or shoots over time. Using an electronic position-sensing transducer to measure the length of intact pea seedlings, Warner and Leopold (1971) found that application of various hormones caused growth inhibition, with latent times ranging from approximately 5 min for abscisic acid to 24 min for gibberellic acid. Application of 10 μl l^{-1} ethylene caused growth inhibition with a latent time averaging approximately 6 min. Unlike later studies by others, they reported that when seedlings were treated with a lower dose of ethylene, the latent time for growth inhibition increased. Burg (1973) used a cathetometer to measure growth and found that 100 nl l^{-1} ethylene started to inhibit the growth of pea roots and shoots, as well as cabbage shoots, within 15 min. Similar latent times for growth inhibition were reported by Goeschl and Kays (1975) using manual time-lapse photography on pea epicotyls, Rauser and Horton (1975) using a root auxanometer to study pea roots, and Jackson (1983) using a traveling microscope to examine the growth of radish roots. Several of these workers also examined the growth recovery kinetics after removal of ethylene. Most found the latent time for growth recovery to be approximately 15–20 min over a range of ethylene concentrations (Warner and Leopold 1971; Burg 1973; Jackson 1983). However, a much longer latent time of 1–2 h for growth recovery was observed in pea roots treated with 1 ml l^{-1} ethylene (Rauser and Horton 1975).

For completeness, it should be mentioned that one group used time-lapse photography to study the rapid effects of ethylene on gravitropic bending of maize roots and reported that ethylene delayed the onset of gravitropic bending (Lee and others 1990). However, ethylene-treated seedlings responded to gravity for a longer time than seedlings not treated with ethylene. This resulted in a greater final curvature of bending in the ethylene-treated seedlings. Consistent with this, inhibitors of ethylene synthesis or action reduced both the latent time for gravitropic bending and the maximum bend due to gravity. They found that these effects of ethylene on root gravitropism most likely involved alterations in auxin transport. Thus, kinetic analysis can be used in a number of ways to study ethylene responses.

Although many of the details obtained from these growth kinetic studies are not identical, they provided information about the timing of the effects of ethylene on growth regulation. In particular, they showed that ethylene could cause growth inhibition in a variety of species within 10 min and that growth recovery starts approximately 15–20 min after ethylene treatment stops. With this information, it was possible to correlate the

timing of growth changes at the organ level with the effects of ethylene on other processes such as cell division, cell expansion, DNA and RNA synthesis, and cellulose microfibril orientation (Burg 1973). However, the tools necessary to dissect the ethylene signaling pathway were not available for these studies. The advent of *Arabidopsis* as a model system with a sequenced genome and a large number of mutants available has made it possible to analyze the ethylene signaling pathway. This progress in genetics and molecular biology, coupled with advances in technology that make automated time-lapse imaging possible, provides an opportunity to use this kinetic approach to uncover new details about ethylene signaling. Two recent studies out of the laboratory of the late Anthony Bleecker (Binder and others 2004a,b) have used this methodology to do this. The remainder of this review describes the technique used and summarizes these studies.

The Method: High-resolution, Computer-driven, Time-lapse Imaging

Recently, interest in assessing the response kinetics to the application and removal of ethylene has resurfaced. The time-lapse imaging system used to do this was developed in the laboratory of Edgar Spalding at the University of Wisconsin to examine the responses of hypocotyls to light (Parks and Spalding 1999; Folta and Spalding 2001; Folta and others 2003). It was necessary to design the system to have high resolution because of the small size of *Arabidopsis* seedlings. It was also necessary to design the system so that seedlings could be imaged without stimulating them with light. The development of computer-driven digital cameras that were sensitive to wavelengths of light in the infrared range provided the opportunity to make such a system that had the added advantage of being automated.

This system was modified in the laboratory of Anthony Bleecker to study the effects of ethylene on growing, etiolated seedlings. In that system (Figure 1), a digital camera fitted with a close-focus zoom lens is driven by a computer with custom software allowing image acquisition at intervals from milliseconds to hours. The zoom lens allows image resolutions up to 170 pixels mm^{-1}. Seedlings are grown on a vertically orientated agar plate in a sealed chamber, and lighting is provided by an infrared light-emitting diode positioned behind the growing seedlings. Having the seedlings grow along the surface of the agar keeps them in the plane of focus. Overall gas flow is maintained at 100 ml min^{-1} throughout the experiment using digital mass flowmeters and controllers. Gas is introduced into the chamber through an inlet fitted into the lid of the chamber and an outlet in the lid allows for gas outflow from the chamber. Ethylene can be applied in precise concentrations, and under these conditions equilibration occurs in under 5 min (Binder and others 2004a,b). The length of roots or shoots in pixels can be measured in each frame manually or with custom software developed by Edgar Spalding (Folta and Spalding 2001). From these measurements, the growth rate can be calculated.

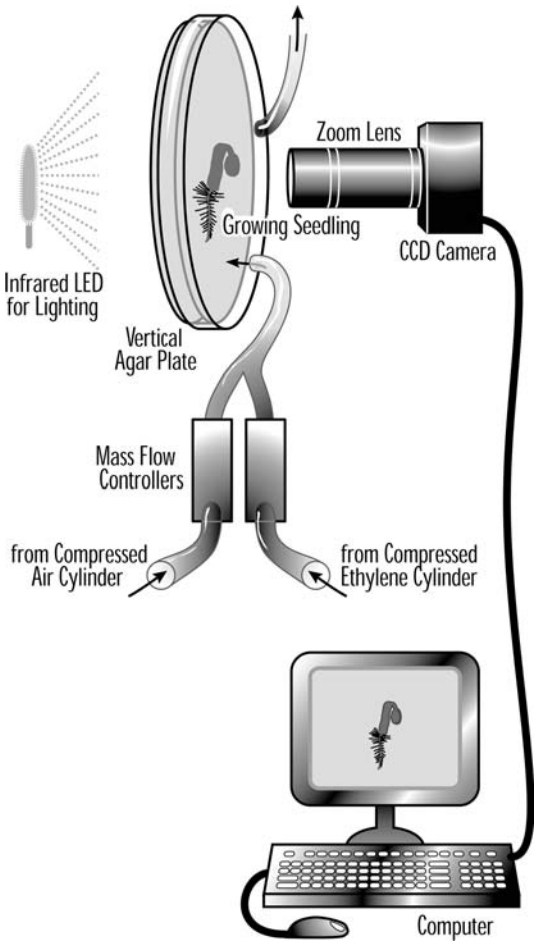

Figure 1. Schematic diagram of computer-driven, high-resolution, time-lapse imaging system. To measure the growth rate of etiolated plants, seedlings are grown on a vertically orientated agar plate. A computer-driven, charge-coupled device (CCD) camera fitted with a close-focus zoom lens is used to acquire digital images. Back lighting is provided by an infrared LED, and images are stored in a computer for analysis. Ethylene concentration is controlled with mass flowmeters and controllers. Arrows show direction of gas flow through the system (drawing by Kandis Elliot).

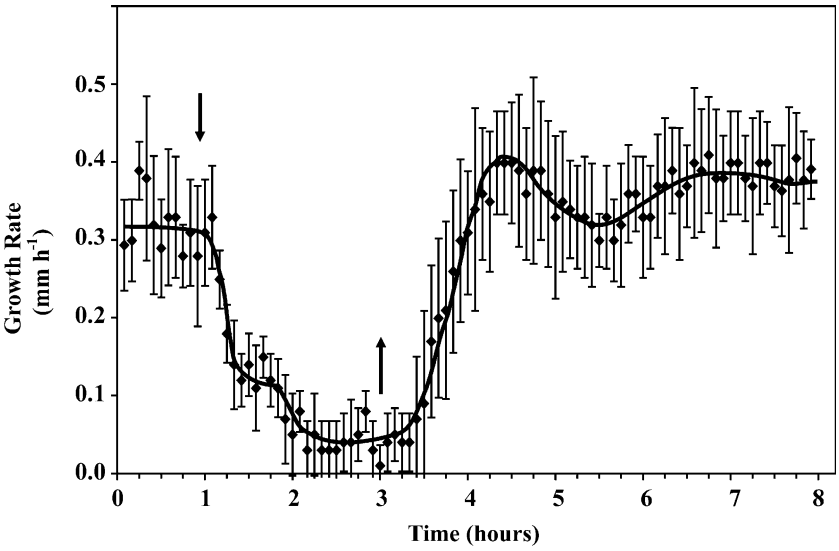

Figure 2. Rapid growth response kinetics of the hypocotyls of etiolated *Arabidopsis* seedlings to the application and removal of ethylene. Measurements were made in air for 1 h prior to introducing 10 µl l^{-1} ethylene (↓). Ethylene was removed 2 h later (↑). Images were captured every 5 min. Seedlings were grown in the presence of AVG to block biosynthesis of ethylene. The average growth rate ± SD is shown, and the line is fitted to data by hand (modified from Binder and others 2004b).

Ethylene Response and Recovery Kinetics

Two articles out of the laboratory of the late Anthony Bleecker have used this system coupled with mutational analysis to address questions about the roles of the ethylene receptors, receptor histidine kinase activity, and downstream components in ethylene signaling (Binder and others 2004a,b).

These researchers found that hypocotyls from etiolated wild-type *Arabidopsis* seedlings responded rapidly to exogenous ethylene reaching a new steady-state growth rate about 75 min after the application of ethylene (Figure 2). After the withdrawal of ethylene from the treatment chamber, hypocotyls that had been growing in the presence of ethylene for 2 h began to recover within 25 min, attaining pretreatment growth rates approximately 90 min after removal of ethylene. Often, a dampened oscillation in growth rate was observed after recovery (Figure 2). Growth rates do not appear to correlate with the ethylene response kinetics. For instance, unpublished results showed that in air, *Arabidopsis* roots grow more slowly than hypocotyls, yet both have similar early response kinetics to the addition of ethylene (Figure 3) (B. M. Binder and A. B. Bleecker, unpublished results). However, other species have different growth-response kinetics when measured with this system. For instance, etiolated tomato (German Queen) shoots grew faster than *Arabidopsis* hypocotyls but recovered more slowly after the removal of ethylene (Figure 4).

Closer examination of the response kinetics of *Arabidopsis* hypocotyls by these workers uncovered two phases of growth inhibition by ethylene. The first, a rapid deceleration phase, had a lag of approximately 10 min after ethylene was applied (Figures 2, 3, and 5). Unlike the results of Warner and Leopold (1971), but similar to those of Goeschl and Kays (1975), this delay in growth inhibition appeared to be independent of ethylene concentrations between 1.5 nl l^{-1} and 10 µl l^{-1} (Binder and others 2004a). The underlying mechanism for this delay is not clear, but it is not an intrinsic characteristic of hypocotyls given that blue light can cause growth inhibition of *Arabidopsis* hypocotyls with a much shorter delay (Parks and others 1998).

The first phase of growth inhibition lasted approximately 15 min and resulted in an intermediate steady-state growth rate. The growth rate remained stable for approximately 25 min before a second, slower phase of growth inhibition followed. The rate of change for this slower phase was approximately 1/6 the rate of change observed during the first phase. This lasted another 20 min until the growth rate reached a new, lower steady-state rate. At ethylene concentrations 1 µl l^{-1} or above, the growth rate was suppressed to this low level until ethylene was removed. However, in the continued presence of intermediate levels of ethylene (between approximately 10 nl l^{-1} and 1 µl l^{-1}) a third phase to the growth response was revealed where the growth rate slowly increased to intermediate rates starting approximately 2–2.5 h after the addition of ethylene (Figure 5).

These observations show that *Arabidopsis* seedlings can respond quickly to the application and removal of ethylene and that the growth-inhibition response is complex.

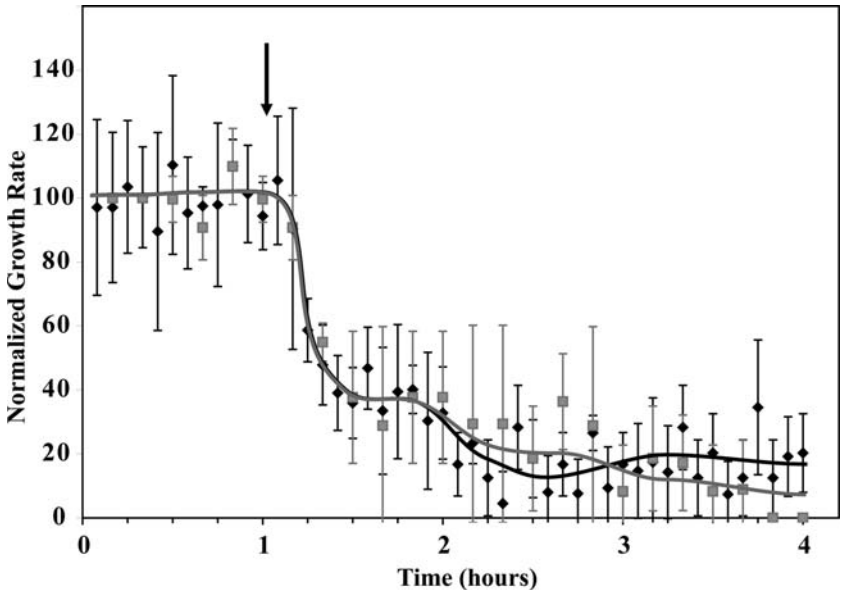

Figure 3. Root and shoot growth inhibition kinetics in the presence of ethylene. Responses of hypocotyls (black) are compared to those of roots (gray). Columbia (wt) seedlings were grown in air for 1 h prior to addition of 10 μl l^{-1} ethylene (↓). AVG was omitted from these assays since it slows root growth (Larsen and Chang 2001). Images for hypocotyl measurements were taken every 5 min, and those for roots were taken every 10 min. The rate of growth was determined throughout and normalized to the air pretreatment conditions. The average normlized rates ± SD are shown, and lines are fitted to data by hand (unpublished results).

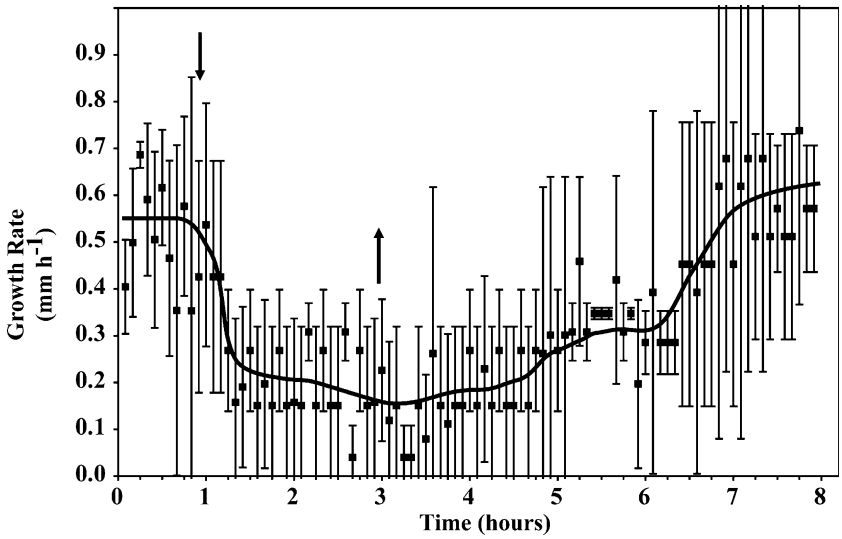

Figure 4. Rapid growth response kinetics of the shoots of etiolated tomato seedlings to the application and removal of ethylene. Measurements were made under conditions identical to those in Figure 2, except a lower magnification was used to accommodate the larger size of the seedlings (unpublished results).

What Rapid Kinetic Studies Tell Us About Receptor Function

In the first of two articles, Binder and others (2004b) focused on receptor function in regulating growth. An overview of ethylene receptors is given here to provide some context for their work. Plants respond to ethylene over a wide concentration range (Chen and Bleecker 1995; Binder and others 2004a). According to current models, responses to ethylene are mediated by a family of receptors that have homology to bacterial two-component receptors. In *Arabidopsis* there are five receptor isoforms (ETR1, ERS1, ETR2, EIN4, ERS2) all of which can bind ethylene (Schaller and Bleecker 1995; Hall and others 2000; O'Malley and others 2005). Specific mutations in any of these isoforms confer dominant ethylene insensitivity on the plant (Bleecker and others 1988; Chang and others 1993; Hua and Meyerowitz 1995; Hua and others 1998; Sakai and others 1998; Hall and others 1999). Genetic studies indicate that the receptors have overlapping but distinct roles in signaling, whereby the receptors are negative regulators of ethylene responses (Hua and Meyerowitz 1998; Zhao and others 2002; Hall and others 2000; Wang and others 2003; O'Malley and others 2005). The receptors can be divided into two subfamilies. Subfamily I consists of ETR1 and ERS1, which contain all amino acid residues needed for His kinase activity (Chang and others 1993; Hua and others 1995) and show His kinase activity

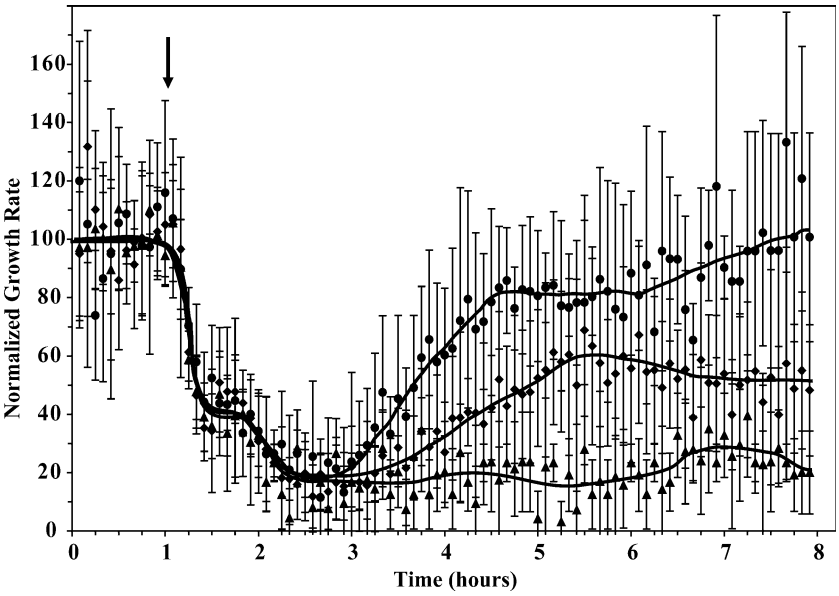

Figure 5. Prolonged responses to ethylene, but not initial growth kinetics, are dose-dependent. Hypocotyl growth response kinetics in seedlings treated with 10 (▲) 0.1 (♦), and 0.05 (●) μl l^{-1} ethylene. Seedlings were grown in air for 1 h before ethylene was introduced (↓). Growth was normalized to growth rates during the air pretreatment. The average growth rates ± SD are shown, and lines are fitted to data by hand (Adapted from Binder and others 2004a).

in vitro (Gamble and others 1998; Moussatche and Klee 2004). Subfamily II includes ETR2, EIN4, and ERS2, which contain degenerate His kinase domains (Hua and others 1998; Sakai and others 1998) and have Ser/Thr kinase activity *in vitro*. ERS1 is capable of both His and Ser/Thr kinase activities *in vitro* depending on the assay conditions used and is likely to have Ser/Thr kinase activity *in vivo* (Moussatche and Klee 2004). Three of these isoforms (ETR1, ETR2, EIN4) contain a receiver domain. Although the kinase domain of ETR1 appears to be required for signalling (Qu and Schaller 2004), kinase activity *per se* is not (Wang and others 2003; Binder and others 2004b; Qu and Schaller 2004). In the evolutionarily related bacterial systems, two-component receptors transduce signal via the autophosphorylation of a His residue in the kinase domain, followed by the transfer of phosphate to a conserved Asp residue in the receiver domain of a response regulator protein (West and Stock 2001). It remains an open question what role each receptor isoform has in ethylene signaling and what, if any, roles His kinase and Ser/Thr kinase activities play in receptor function.

The role of each receptor isoform in the kinetics of ethylene growth responses was examined using receptor loss-of-function mutants (Binder and others 2004b). None of the loss-of-function mutants, either singly or in combination, had a measurable effect on the first two phases of growth inhibition indicating redundant receptor function during the initial responses to ethylene. In contrast, loss-of-function mutants in receptor isoforms containing a receiver domain (ETR1, ETR2, EIN4) prolonged growth recovery after the removal of ethylene. A triple mutant lacking all three receptor isoforms took much longer to recover than any of the single mutants, whereas mutations in both *ERS1* and *ERS2* had no measurable effect on growth recovery. Together these findings suggest a role for receptors containing a receiver domain in growth recovery of etiolated seedlings after removal of ethylene.

To determine if this was linked to histidine kinase activity, growth recovery was examined in the histidine kinase–deficient, receptor subfamily I double mutant. Etiolated seedlings with this double mutant had previously been shown to have slow growth in air. In light, these mutants displayed a number of severe growth phenotypes, including small rosettes, delayed flowering, and sterility (Hall and Bleecker 2003). Growth kinetic analysis showed that the double mutant had delayed growth recovery, similar to the single *etr1-7* mutant (Binder and others 2004b). Transformation of the double mutant with a wild-type *ETR1* transgene rescued the slow recovery phenotype, whereas a histidine kinase–inactivated construct did not. In contrast, both the wild-type and histidine kinase–inactivated constructs rescued growth in air as well as the other growth phenotypes exhibited in light (Wang and others 2003; Binder and others 2004b). These findings indicate that ETR1 His kinase activity is not needed for ethylene signaling but is involved in normal growth recovery after stimulation by ethylene.

In a second paper, Binder and others (2004a) used a pharmacological approach to further characterize the two phases of growth inhibition and

uncovered more details about receptor signaling. They found that the first phase growth response was much less sensitive to the ethylene response inhibitor 1-methylcyclopropene (1-MCP) than the second phase response. This inhibitor is modeled to work by binding to the active site of the receptor, thus blocking ethylene binding (Binder and Bleecker 2003). Consistent with this, the first phase growth inhibition response is much more sensitive to ethylene. A phase I growth inhibition response continued to be present at concentrations from 2 nl l^{-1} (the approximate threshold for the phase II growth response) down to 0.2 nl l^{-1} ethylene, which is well below the published K_d for yeast-expressed ETR1 (Schaller and Bleecker 1995). The phase I growth response was transient and became shorter in duration with lower ethylene concentrations. Like bacterial chemotaxis, the phase I growth response showed adaptation at low doses of agonist ($\leq$ 10 nl l^{-1} ethylene). This adaptation had a relative refractory period of 5 h.

Based on the published K_d of 2.4 nM for the ETR1 receptor (Schaller and Bleecker 1995), the phase I response is occurring when approximately 1 out of 1,000 receptors are turned off by ethylene. This ability to respond to small changes in ethylene concentration is reminiscent of the behavior associated with the evolutionarily related bacterial two-component receptors (Thomason and other 2002). Models for bacterial chemotaxis propose that amplification results from receptor dimers forming clusters, where the occupancy state of one dimer can shift the signaling states of surrounding receptor dimers within a cluster through physical interaction (Bray and others 1998; Duke and Bray 1999; Shimizu and others 2003). The consequence of this is that changes in receptor occupancy at low concentrations are amplified, leading to a large change in total receptor output. There is no direct evidence for ethylene receptors forming clusters, but this has been put forth as a possible explanation for insensitivity of dominant mutant forms of receptors (Gamble and others 2002).

The rate of growth recovery also suggests that receptor clustering might play a role in growth recovery. It is thought that receptor output for long-term growth inhibition is directly proportional to receptor occupancy (Schaller and Bleecker 1995). Thus, recovery to pretreatment growth rates is predicted to occur when the majority of receptors have reverted to the active, unbound state. This can occur by dissociation of ethylene from the receptors or, once exogenous ethylene has been removed, by synthesis of new receptors. Presumably, both processes are contributing to this shift to an unbound, active receptor. However, existing evidence suggests that ethylene release is much slower than growth recovery (Schaller and Bleecker 1995; O'Malley and others 2005). Because ethylene release would be a function of both ethylene dissociation from intact receptors and release from receptors that are being broken down, the bulk degradation rate for receptors also appears to be on a slower time scale in plants (Sanders and others 1991). Additionally, the longevity of the effects of 1-MCP, a competitive inhibitor of ethylene binding (Hall and others 2000) and responses (Blankenship and Dole 2003), indicates that ethylene receptor turnover is too slow to explain growth recovery in a simple model. A model in which receptors act cooperatively is one way to reconcile the relatively slow changes in receptor occupancy, with the more rapid changes in response output leading to growth recovery. Although signal amplification could also occur via a multi-step signal cascade, the demonstration that histidine-kinase activity and the receiver domains are required for maximum recovery rates suggests that receptors play some direct role in amplification of signal during recovery.

Kinetic Studies and Downstream Signalling

Rapid growth kinetic measurements have also led to a more refined understanding of signaling that occurs downstream of the receptors. Current models for ethylene signaling are largely linear. In these models the five ethylene receptors form a complex with a kinase, CTR1, that negatively regulates response pathways in the absence of ethylene (Kieber and others 1993; Huang and others 2003; Gao and others 2003). There is genetic evidence that ethylene responses require the presence of the membrane protein EIN2 (Alonso and others 1999). Ethylene binding inhibits the receptor/CTR1 complex leading to an increase in activity of EIN2 protein along with subsequent signaling associated with it. Two targets downstream of EIN2 are the EIN3 and related EIL1 transcription factors that are required for long-term ethylene responses (Chao and others 1997; Alonso and others 2003). Several recent reports showed that ethylene leads to an increase in the protein levels of the EIN3 transcription factor (Yanagisawa and others 2003; Guo and Ecker 2003; Potuschak and others 2003; Gagne and others 2004). In the absence of ethylene, EIN3 was rapidly degraded by the ubiquitin/26S proteasome pathway using an

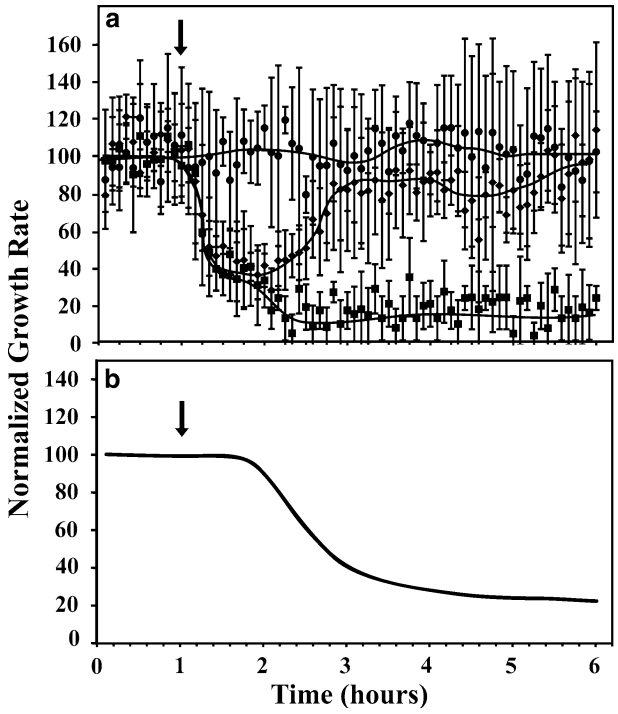

Figure 6. Phase I and phase II growth inhibition can be distinguished genetically. **A**. Growth responses of Columbia wild-type (■), *ein3-1 eil1-1* double mutants (♦), and *ein2-1* mutants (●) are shown. Seedlings were grown in air for 1 h before 10 μl l^{-1} ethylene was introduced (↓). Growth was normalized to growth rates during the air pretreatment. **B**. The predicted shape of the phase II response was calculated by subtracting the response of the *ein3 eil1* double mutants from the wild-type response. Lines were fitted to data by hand (panel A modified from Binder and others 2004a).

SCF E3 complex containing the EBF1 and EFB2 F-Box proteins for selective ubiquitination (Guo and Ecker 2003; Potuschak and others 2003; Gagne and others 2004). It is likely that EIL1 is also a target of the $SCF^{EBF1/2}$ complexes (Potuschak and others 2003). Ethylene treatment blocks this ubiquitination via EIN2, resulting in the increase in EIN3 levels. It is unknown whether this inhibition is caused by an alteration of the EIN3/EIL1 substrates or of the $SCF^{EBF1/2}$ complexes or both. Ethylene also stimulated an EIN3-dependent increase in the transcript levels of *EBF2* and to a lesser extent *EBF1* (Potuschak and others 2003; Guo and Ecker 2003; Gagne and others 2004). This could function to limit the magnitude of ethylene responses and is likely to be critical for the survival of the plant given the very high levels of EIN3 that accumulate and the extreme developmental arrest that occurs when both *EBF1* and *EBF2* are eliminated (Gagne and other 2004).

Binder and others (2004a) focused on the roles of EIN2, EIN3, and EIL1 in the kinetics of the ethylene growth response. EIN3 and EIL1 are required for prolonged responses to ethylene, as evidenced by the observation that the *ein3-1 eil1-1* double loss-of-function mutant had no responses to long ethylene treatments using end-point analysis of growth (Alonso and others 2003). Surprisingly, when studied with time-lapse imaging, this double mutant was found to have the transient phase I response but lacked the prolonged phase II response (Figure 6A; Binder and others 2004a). During the first 30 min after the addition of ethylene, the double mutant response was indistinguishable from the wild-type response. However, after the initial plateau in growth rate, the mutants behaved differently from the wild-type seedlings and showed an acceleration in growth rate in the continued presence of ethylene. Mutant *ein2-1* seedlings showed no transient growth responses to ethylene (Figure 6A). By subtracting the growth responses of the *ein3 eil1* double mutant from the wild type, it is possible to generate a putative shape for the phase II growth response (Figure 6B). From such analysis, a delay of approximately 1 h after the addition of ethylene is predicted for this second phase response. Thus, the first phase response is EIN3/EIL1-independent, fast in onset, and transient in nature. In contrast, the second phase requires EIN3/EIL1, is slower in onset, and is prolonged while seedlings are maintained in high concentrations of ethylene.

Further evidence of a central role for EIN3 in prolonged ethylene responses was provided by recent studies on *XRN4*. This 5′ → 3′ exoribonuclease, which is allelic to *EIN5* and *EIN7*, was identified as a component downstream of CTR1 that regulates the levels of *EBF1* and *EBF2* (Olmedo and others 2006; Potuschak and others 2006). Mutations in this gene led to increased accumulation of *EBF1* and *EBF2*, resulting in reduced levels of EIN3 protein and partial ethylene insensitivity (Guo and Ecker 2003; Olmedo and others 2006; Potuschak and others 2006). As predicted, rapid kinetic analysis showed that these reduced levels in EIN3 correlated with a more rapid growth recovery after ethylene removal (Potuschak and others 2006). The exact role of *XRN4* in ethylene signaling remains to be determined.

These rapid kinetics data suggest that ethylene signaling is not a simple linear process. Figure 7 shows two possible models that invoke feedback mechanisms to explain the transient nature of the phase I response and the dependence of the phase II response on the EIN3 and EIL1 transcription factors (Binder and others 2004a). In model I (Figure 7),

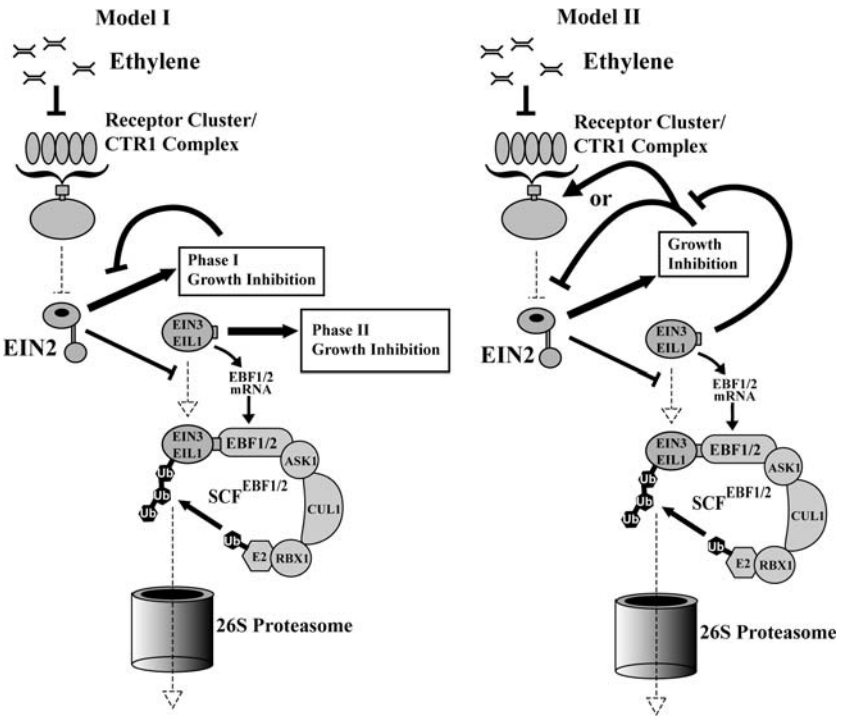

Figure 7. Nonlinear models of ethylene signal transduction. There are two models of ethylene signaling for the first two phases of growth inhibition. Feedback is invoked in both models to explain the adaptation of phase I. (Modified from Binder and others 2004a.)

EIN2 controls the two phases of growth inhibition independently: the first EIN3/EIL1-independent phase I response and the second, EIN3/EIL1-dependent phase II response. In this model, there is negative feedback at or downstream of EIN2 to reverse the phase I growth inhibition. In contrast, both phases of growth inhibition may be controlled by a single EIN2-dependent mechanism, as shown in Model II (Figure 7). In this second model, there is a primary feedback loop that could act to negatively regulate EIN2 or to positively regulate the receptor/CTR1 complex, leading to growth recovery in the presence of ethylene. The function of EIN3 and EIL1 in this case is to provide negative feedback on this primary feedback pathway so that the growth response remains prolonged. Because EIN3 and EIL1 are not required for the phase I response, it remains unclear whether the phase I growth response is controlled at the level of gene expression. It is possible that other members of the EIN3 family of transcription factors (EIL2–5) are involved. Alternatively, other cellular processes might lead to the initial, rapid growth inhibition.

The Kinetics of Other Responses to Ethylene

Time-lapse imaging makes it possible to examine the rapid kinetics of other responses to ethylene,

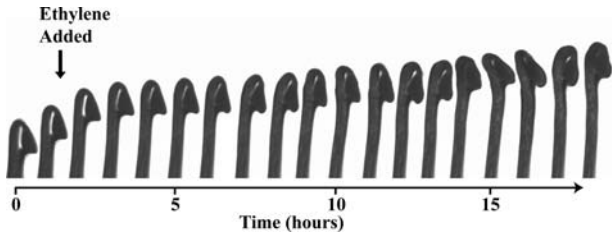

Figure 8. Ethylene stimulates nutational bending of *Arabidopsis* hypocotyls. A series of images were taken at 1-h intervals of hypocotyls of etiolated *Arabidopsis* seedlings in air and at various times after the addition of 10 μl l^{-1} ethylene (unpublished results).

such as increased radial expansion of the hypocotyl and increased tightening of the apical hook. Increased lateral expansion appears to have kinetics similar to those of growth inhibition in excised pea epicotyls (Nee and others 1978; Eisinger and others 1983). However, very little effort has gone into studying this in intact seedlings (Eisinger and others 1983). The maintenance of the apical hook is likely controlled by many factors (Li and others 2004; de Grauwe and others 2005). Detailed kinetic studies of ethylene-stimulated apical hook closure coupled with the use of relevant mutants could uncover new aspects of these interactions. Interestingly, while the author was examining these responses, it was noted that ethylene stimulated nutational bending in the hypocotyls of

etiolated *Arabidopsis* seedlings with an average delay of 6 h (Figure 8; Binder and others 2006). Nutations are oscillatory ''nodding'' or bending movements caused by localized differential growth (Berg and Peacock 1991); they are often termed ''circumnutations'' (Darwin and Darwin 1880). Binder and others (2006) found that the nutation response involves CTR1, EIN2, EIN3, and EIL1. However, unlike growth inhibition, ethylene-stimulated nutations require the ETR1 receptor. Loss of function in this receptor isoform eliminated the nutation response; the nutation phenotype was rescued when these mutants were transformed with a genomic *ETR1* transgene. In contrast, loss-of-function mutations in the other receptor isoforms led to constitutive nutations in air but did not alter ethylene-stimulated nutations. These results support a model where all the receptors are involved in ethylene-stimulated nutations, but the ETR1 receptor is required and has a contrasting role from the other receptor isoforms in this nutation phenotype. These observations open up the possibility of studying ethylene signaling in a new context that is likely to uncover novel details about the signaling pathway.

Future Directions

Kinetic analysis of seedling growth has provided information about the short-term responses to the application and removal of ethylene. This information, coupled with the use of mutants in ethylene signalling, has uncovered new details about the ethylene transduction pathway that would have remained unknown using only end-point analysis.

The recent observation that ethylene stimulates nutations raises the question: ''what other responses to ethylene have yet to be uncovered?'' Although high-resolution, time-lapse imaging can be used to examine the kinetics of various responses, it has drawbacks that make doing this difficult. Two drawbacks are that data analysis is time consuming and only a small number of seedlings can be imaged in an experiment. Even though analysis of growth is fairly straightforward, analyzing changes in apical hook curvature and bending of the hypocotyl or root are more complex and difficult to do manually. One way to overcome this difficulty is to automate the process. Efforts are underway in the Spalding laboratory to write computer algorithms that extract shape and size information from images (Miller and Spalding, personal communication). Such automated morphometric analysis should make it possible to follow growth rates more easily—apical hook curvature, as well as bending of the root and shoot. With higher resolution cameras now available, it is possible to adequately image more seedlings at a time. This automated tracking of multiple responses coupled with the ability to image more seedlings in an experiment opens up the possibility of following the growth and movement of many seedlings in a single experiment. Additionally, this will make it possible to screen seedling populations for subtle ethylene response mutants.

Even without these advances, there are still many details about ethylene signaling that can be examined with rapid kinetic analysis. Clearly, rapid kinetic analysis will be needed to understand the mechanisms for the first phase of growth inhibition (Figures 2, 3), the dampened oscillation observed upon growth recovery after removal of ethylene (Figure 2), and the third phase of the growth response that is observed at intermediate ethylene levels (Figure 5). Kinetic growth analysis will also likely be helpful in refining our understanding about the roles of known components in the ethylene signaling pathway. For instance, the EBF1/2 F-box proteins appear to have distinct but overlapping roles in regulating growth, as evidenced by the small constitutive growth response in air and enhanced responsiveness at low ethylene concentrations observed in *ebf1* mutants and enhanced response to higher levels of ethylene observed in *ebf2* mutants (Guo and Ecker 2003; Gagne and others 2004). Additionally, the kinetics of EIN3 protein accumulation are different in the two mutants compared to wild-type plants, where EIN3 accumulated faster in *ebf1* mutants and to higher levels but at the same rates in *ebf2* mutants (Gagne and others 2004). Given that the long-term, phase II growth response appears to be directly linked to the levels of EIN3 and EIL1 (Alonso and others 2003; Binder and others 2004a), several predictions can be made about the growth-response kinetics of the *ebf1* and *ebf2* mutants. The *ebf1* mutants should have a faster onset of the phase II growth inhibition than wild-type seedlings, whereas the *ebf2* mutants should have wild-type onset kinetics but slower growth rates during phase II growth inhibition or slower recovery after ethylene is removed or both. Because the *ebf2* mutants accumulate higher levels of EIN3, it is likely that plants overexpressing EIN3 will have similar response kinetics to the *ebf2* mutants. Testing these predictions with rapid growth kinetic measurements could provide new insights into the roles of EBF1 and EBF2.

In addition to kinetic studies on *Arabidopsis* seedlings, time-lapse imaging of etiolated seedlings can be applied to other species (Figure 4). Although the components of ethylene signal transduction are similar between *Arabidopsis* and other species, they are not identical (Klee 2004). The increased number of ethylene-related mutants in other species makes them ripe for these rapid growth kinetic studies. Coupling kinetic studies with mutants in these other species could uncover new details about seedling responses leading to more detailed models of ethylene signalling in a variety of species.

Clearly, many approaches are required to fully understand ethylene signaling and responses. As greater numbers of mutants with subtle phenotypes become available, scientists will need more refined methods of analysis. The time-lapse imaging system described in this review is one approach that will undoubtedly be of great use in the future.

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