BMC Plant Biology

RESEARCH Open Access

Modulation of plant growth and development through altered ethylene binding affinity of the ethylene receptor ETR1

Sitwat Aman[1], Swadhin Swain[1], Esha Dutta[2], Safdar Abbas[1,3], Ning Li[3], Samina N. Shakeel[1,4], Brad M. Binder[5] and G. Eric Schaller[1*]

Abstract

Arabidopsis senses ethylene through a five-member family of ethylene receptors, of which the ethylene receptor ETR1 plays the major role. We examined how changes in ethylene binding affinity of ETR1 can regulate physiological and molecular responses to ethylene, taking advantage of an Asp25Asn mutation that still produces a functional ETR1 receptor ($ETR1^{D25N}$) but one with 100-fold reduced ethylene binding affinity compared to wild-type ETR1 ($ETR1^{wt}$). Analysis was performed in a genetic background that lacks the five native members of the receptor family so that the specific role of ETR1 in plant growth and development could be assessed. From this analysis, we determined that changes in ethylene binding affinity of ETR1 are reflected in plant growth and responses to ethylene. Differences in plant growth and ethylene responses for the $ETR1^{wt}$ and $ETR1^{D25N}$ lines were uncovered in seedlings grown under light or dark conditions, and when assayed for short- or long-term responses to ethylene. Dose response analysis revealed that differences in the ethylene responses for $ETR1^{wt}$ and $ETR1^{D25N}$ lines are proportional to the binding affinity of the corresponding receptor variants. Results from the characterization of the $ETR1^{wt}$ line and an *etr1 etr2 ein4* triple mutant demonstrate that plants have greater sensitivity to ethylene than previously recognized.

Keywords Arabidopsis, Phytohormone, Ethylene, Ethylene receptor, Ligand binding

Introduction

The gaseous phytohormone ethylene regulates multiple aspects of plant growth and development [1, 2]. In addition to its well-known role in the ripening, ethylene regulates seed germination, root and shoot growth, leaf and petal abscission, senescence, and responses to biotic and abiotic stresses. Plants are remarkably sensitive to ethylene. For example, ethylene concentrations as low as 0.2 nL L^{-1} inhibit growth of dark-grown Arabidopsis seedlings [3]. At the other extreme, differential responses to ethylene have identified at concentrations as high as 1000 µL L^{-1} ethylene, an example being the induction of a chitinase-B-glucuronidase reporter in Arabidopsis stems [4]. As such, Arabidopsis is capable of sensing

*Correspondence:
G. Eric Schaller
george.e.schaller@dartmouth.edu
[1]Department of Biological Sciences, Dartmouth College, Hanover, NH 03755, USA
[2]Genome Science and Technology Program, University of Tennessee, Knoxville, TN 37996, USA
[3]Division of Life Science, The Hong Kong University of Science and Technology,, Hong Kong, SAR 518057, China
[4]Department of Biochemistry, Quaid-i-azam University, Islamabad 45320, Pakistan
[5]Department of Biochemistry & Cellular and Molecular Biology, University of Tennessee, Knoxville, TN 37996, USA

and responding to ethylene concentrations that span six orders of magnitude [5].

Ethylene is sensed in plants by receptors evolutionarily related to bacterial His-kinases, the first identified and most thoroughly characterized of the ethylene receptors being ETHYLENE RESPONSE 1 (ETR1) of Arabidopsis [6–10]. Ethylene receptors have an ethylene-binding domain located near their N-terminus and signal output domains in their C-terminal portion. The ethylene-binding domain is located within three conserved transmembrane segments, which also serve to localize the receptors to the ER membrane [7, 11–16]. A critical component of the ethylene binding domain is a copper cofactor chelated by conserved Cys and His residues. The copper cofactor directly mediates ethylene binding, there being no amino-acid sidechains compatible with the such high-affinity binding as found with the ethylene receptors [11, 17, 18]. Current studies indicate that there is one copper cofactor per receptor monomer, resulting in two copper cofactors per receptor homodimer [11, 19–21]. The output domains of the ethylene receptors are similar to those found in bacterial His-kinases, some receptors also having domains similar to the receiver domains of response regulators [22–24]. However, His-kinase activity of the ethylene receptors only plays a minor role in signaling [25]. Instead, output is largely mediated through interactions of the receptor output domains with the Ser/Thr kinase CONSTITUTIVE ETHYLENE RESPONSE 1 (CTR1), which in turn regulates activity of the transmembrane protein ETHYLENE INSENSITIVE 2 (EIN2), which in turn regulates a transcriptional cascade involving the EIN3/ETHYLENE INSENSITIVE 3-LIKE (EIL) and ETHYLENE RESPONSE FACTOR (ERF) families of transcription factors [9, 10].

Ethylene receptors are found as families in most plants [8, 9]. Arabidopsis, for example, has a five-member family of ethylene receptors composed of ETR1, ETR2, EIN4, ETHYLENE RESPONSE SENSOR 1 (ERS1), and ERS2 [8, 9]. Genetic studies indicate that the ethylene receptors function as negative regulators of ethylene signaling, such that they suppress ethylene responses in the absence of ethylene (in air) and, upon binding ethylene, are inactivated to relieve the suppression [8, 9, 26–28]. Both gain-of-function and loss-of-function mutations have been identified for the Arabidopsis ethylene receptors [8, 9]. The first mutation identified was the gain-of-function mutation *etr1-1* of Arabidopsis, which conferred dominant ethylene insensitivity on the plant [29]. The *etr1-1* missense mutation (Cys65Tyr) directly affects one of the amino acids necessary for chelating the copper cofactor, generating a receptor unable to sense ethylene and, as a result, serving to suppress ethylene responses regardless of whether ethylene is absent or present [6, 7, 11]. In contrast, due to their role as negative regulators of ethylene signaling, loss-of-function mutations involving multiple members of the receptor family (e.g. an *etr1 etr2 ein4* triple mutant) confer a constitutive ethylene-response phenotype [26–28].

We recently uncovered a role for Asp25 of ETR1 in modulating the ethylene affinity of the receptor [30]. Specifically, we found that the ETR1^{Asp25Asn} mutant was still functional as a receptor but had an approximately 100-fold lower affinity for ethylene than the wild-type receptor. Based on molecular modeling and genetic analysis, Asp25 forms a polar bond to a critical His residue (His69) important for chelating the copper cofactor that binds ethylene [30]; mutation of Asp25 to Asn eliminates the polar bond to His69, thereby reducing the affinity of the receptor for ethylene. Here we assess how such differences in ethylene binding affinity affect plant growth and ethylene responses, based on physiological and molecular assays. Results point to the key roles that ethylene binding affinity and receptor levels play in mediating plant responses to ethylene. Results also demonstrate that plants have a greater sensitivity to ethylene than previously recognized.

Results

Generation of transgenic lines to examine the role of ETR1 ethylene-binding affinity in Arabidopsis

We previously determined that the Asp25Asn ETR1 mutant receptor (ETR1^{D25N}) was a functional receptor based on its ability to rescue growth phenotypes of receptor loss-of-function mutants (Fig. 1A) [30]. Furthermore, we also determined that ETR1^{D25N} possessed substantially lower affinity for ethylene than the wild-type ETR1 receptor, approximately 100-fold less based on an ethylene binding assay [30]. We were therefore interested in how such a difference in ethylene binding affinity would manifest itself in plant growth and development. However, because Arabidopsis contains a five-member family of ethylene receptors, differences in the ability of one receptor isoform to perceive and respond to ethylene can be masked by the presence of other wild-type receptor isoforms.

Therefore, to compare the effects of ETR1^{wt} and ETR1^{D25N}, we took the approach of transgenically expressing their genes from the native *ETR1* promoter in an *etr1 etr2 ein4 ers1 ers2* quintuple mutant background. To obtain the quintuple mutant background, we first transgenically expressed *ETR1^{wt}* and *ETR1^{D25N}* in the viable triple-mutant *etr1 etr2 ein4*, by which we successfully obtained both tETR1^{wt} (*etr1 etr2 ein4*) and tETR1^{D25N} (*etr1 etr2 ein4*) lines [30]. We then employed a CRISPR-cas9 approach to mutate the *ERS1* and *ERS2* genes that remained in this background (Fig. 1B). This approach was successful for the generation of tETR1^{D25N} (*etr1 etr2 ein4 ers1 ers2*) mutants, for which we obtained

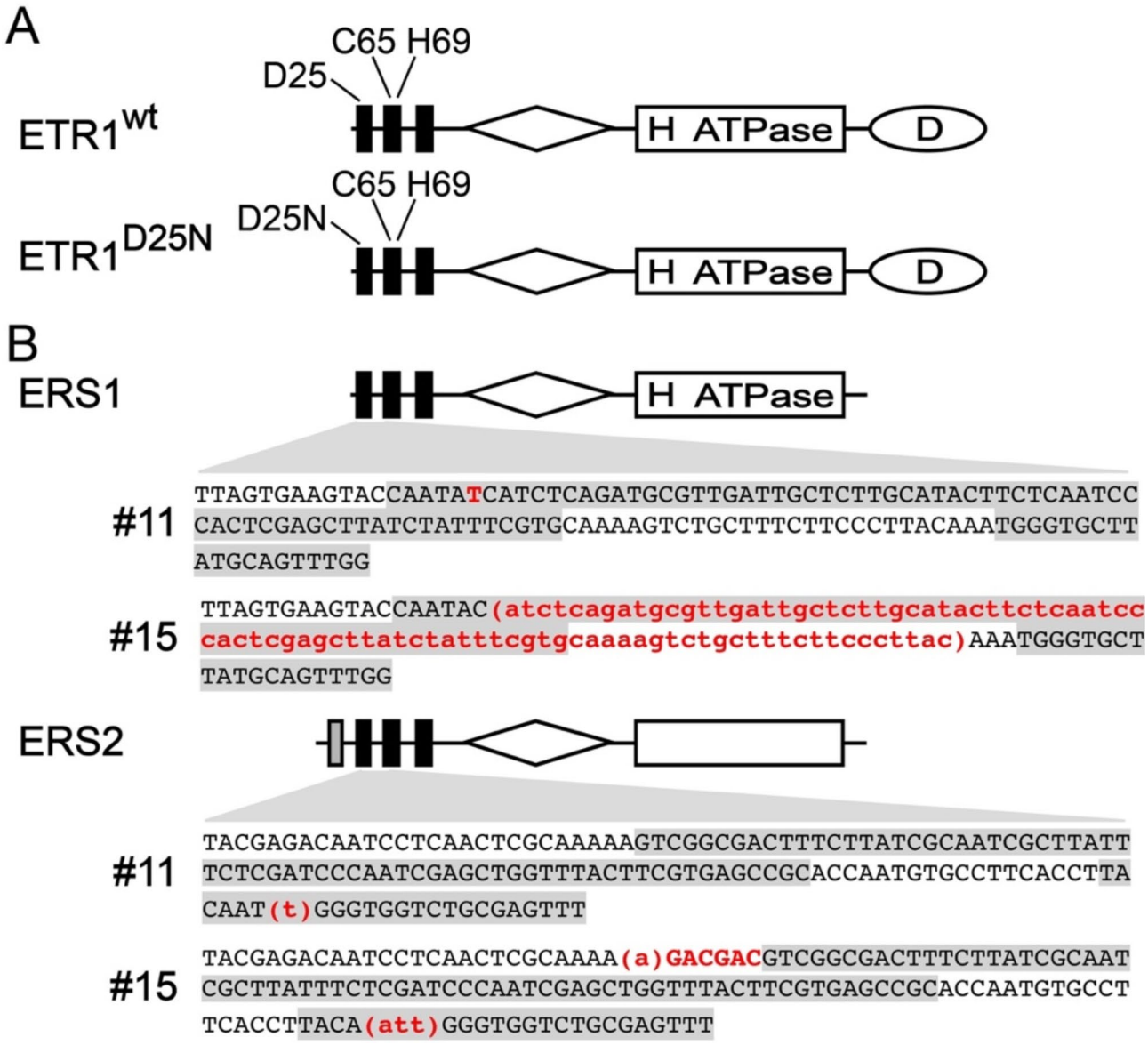

Fig. 1 Characteristics of ethylene receptor mutations. For the receptors, transmembrane domains (black bars), signal peptide (gray bar), GAF domain (diamond), histidine kinase-like domain (rectangle), and receiver domain (oval) are indicated if present. The histidine kinase-like domain is labeled "H ATPase" if it contains all residues required for activity. **(A)** Diagram of wildtype ETR1 (ETR1^{wt}) showing locations of Asp25 (D25), Cys65 (C65), and His69 (H69) involved in ethylene binding and of the mutant ETR1^{D25N}. **(B)** CRISPR-Cas9-derived mutations in ERS1 and ERS2. Uppercase bold red letters indicate insertions. Lowercase bold red letters in parentheses indicate deletions. Gray highlights in the DNA sequence indicate regions that encode transmembrane domains. In the quintuple mutant background *etr1 etr2 ein4 ers1 ers2*, the transgenically-expressed *tETR1^{wt}* line shares the same CRISPR-Cas9-derived mutations in *ERS1* and *ERS2* as found in the transgenically expressed tETR1^{D25N}-#11 line

two independent lines contained indel mutations predicted to result in null alleles for *ERS1* and *ERS2* (lines #11 and #15) [30]. However, we were unable to obtain a tETR1^{wt} (*etr1 etr2 ein4 ers1 ers2*) line by the CRISPR-cas9 approach, generating only a tETR1^{wt} (*etr1 etr2 ein4 ers2*) mutant line that was still wildtype for the *ERS1* allele. Additionally, the *ERS2* mutant allele was a six-base-pair deletion, such that the protein product would still be in frame and potentially not a null allele.

Our inability to obtain the tETR1^{wt} (*etr1 etr2 ein4 ers1 ers2*) line by the direct CRISPR-cas9 aided approach could potentially arise due to this line being less viable than one expressing the tETR1^{D25N} transgene. We therefore crossed the tETR1^{wt} (*etr1 etr2 ein4 ers2*) mutant line to the tETR1^{D25N} #11 (*etr1 etr2 ein4 ers1 ers2*) line and genotyped the subsequent segregating populations to identify the tETR1^{wt} transgene in a quintuple mutant background. By this process, we identified a tETR1^{wt} (*etr1 etr2 ein4 ers1 ers2*) line that contained the same *ers1*

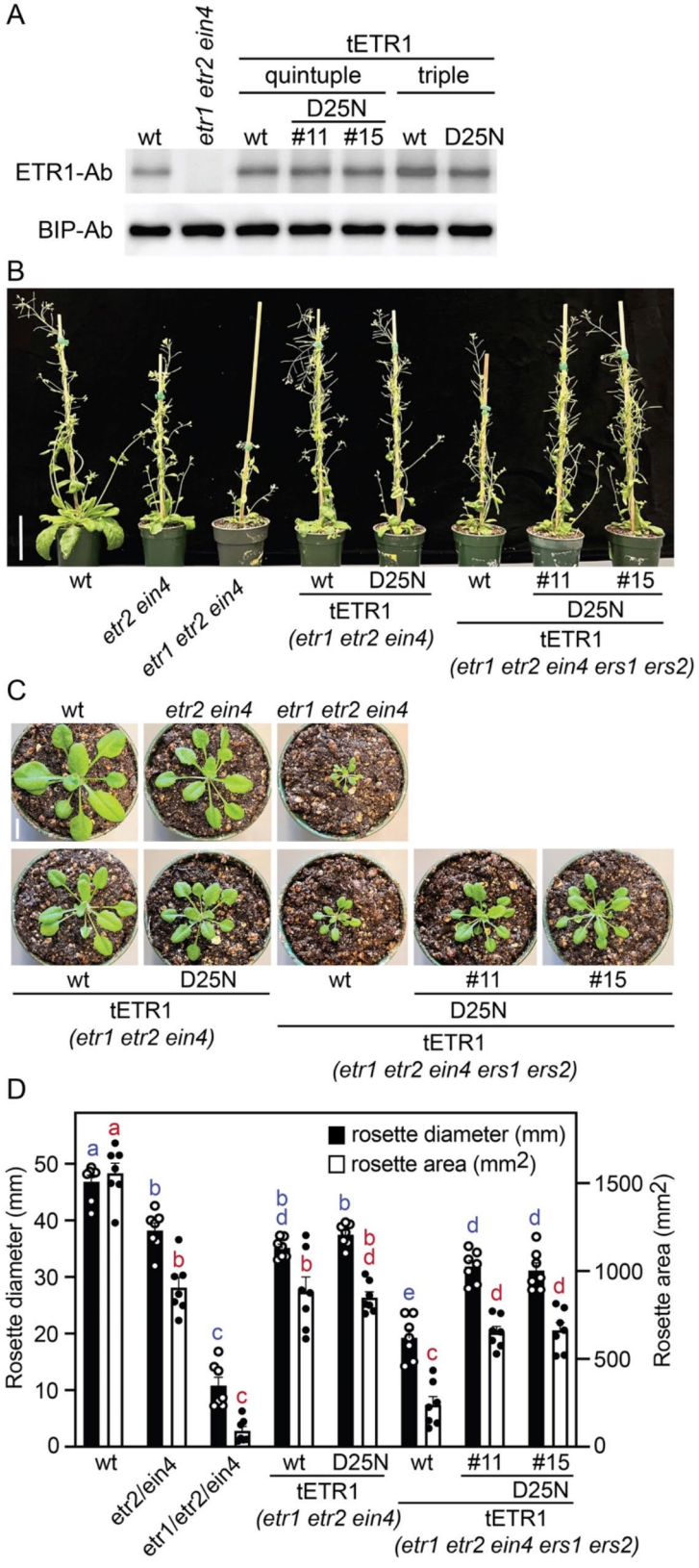

Fig. 2 (See legend on next page.)

(See figure on previous page.)

Fig. 2 Shoot phenotypes of the tETR1^{wt} and tETR1^{D25N} lines. Wildtype and mutant versions of ETR1 were transgenically (tETR1) expressed in the *etr1 etr2 ein4* (triple mutant) and *etr1 ers1 etr2 ers2 ein4* (quintuple mutant) backgrounds. Wildtype, the *etr2 ein4* double mutant, and the *etr1 etr2 ein4* triple mutant serve as controls. **(A)** ETR1 protein levels as determined by immunoblot analysis with an anti-ETR1 antibody for 14-day-old green seedlings. BIP serves as a loading control. **(B)** Representative 45-day-old plants. scale bar = 5 cm. **(C)** Representative 27-day-old shoot rosettes. Scale bar = 1 cm. **(D)** Rosette diameter and area. Different letters indicate a significant difference in diameter (blue letters) or area (red letters) based on ANOVA analysis with post hoc Holm multiple comparisons test ($n = 7$; $p < 0.05$). Error bars represent SE

and *ers2* CRISPR alleles as those found in the tETR1^{D25N} #11 (*etr1 etr2 ein4 ers1 ers2*) line (Fig. 1B).

For the comparative analyses of ETR1^{wt} and ETR1^{D25N} described below, we characterized tETR1^{wt} #4 (*etr1 etr2 ein4*) and tETR1^{D25N} #6 (*etr1 etr2 ein4*) lines, in which the transgenic ethylene receptors are present in a background containing the wild-type receptors ERS1 and ERS2. We also characterized the tETR1^{wt} (*etr1 etr2 ein4 ers1 ers2*) line and two independent tETR1^{D25N} #11 and #14 (*etr1 etr2 ein4 ers1 ers2*) lines, in which the transgenic ethylene receptors are present in a background lacking all the native ethylene receptors.

Effect of ETR1 ethylene-binding affinity on shoot growth

Shoots of the triple mutant *etr1 etr2 ein4* exhibit a partial constitutive ethylene-response phenotype characterized by a smaller leaf rosette and a shorter inflorescence compared to the wild-type plant (Fig. 2) [27]. These phenotypes are rescued similarly by transgenic expression of either ETR1^{wt} or ETR1^{D25N}, the rosette area and diameter being similar to that found in an *etr2 ein4* double mutant (Fig. 2C, D) [30]. Thus, in a background containing the wildtype receptors ERS1 and ERS2, both ETR1^{wt} and ETR1^{D25N} rescue shoot growth to a similar extent. In contrast, when evaluated in a background lacking all five native receptors, the rosette area and diameter of tETR1^{wt} (*etr1 etr2 ein4 ers1 ers2*) is significantly less than that of the tETR1^{D25N} #11 and #14 (*etr1 etr2 ein4 ers1 ers2*) lines (Fig. 2C, D), consistent with a stronger ethylene response in shoots of the tETR1^{wt} (*etr1 etr2 ein4 ers1 ers2*) line. We note that the protein levels of ETR1^{wt} and tETR1^{wt} are similar in the quintuple mutant background (Fig. 2A), indicating that the differences in plant growth (Fig. 2B-D) are due to receptor activity rather than expression level. Furthermore, the severity of the shoot growth phenotype for the tETR1^{wt} (*etr1 etr2 ein4 ers1 ers2*) line compared to the tETR1^{D25N} #11 and #14 (*etr1 etr2 ein4 ers1 ers2*) lines suggests that this phenotype may have compromised our ability to identify such a line in our initial CRISPR-cas9 approach.

Effect of ETR1 ethylene-binding affinity on the triple-response of dark-grown seedlings to ethylene

In response to ethylene, dark-grown seedlings of Arabidopsis exhibit a characteristic 'triple response' phenotype, characterized by inhibition of hypocotyl and root elongation, thickening of the hypocotyl, and the formation of a pronounced apical hook (Fig. 3) [29, 31]. Because they are negative regulators of the ethylene response, receptor loss-of-function mutants such as *etr1 etr2 ein4* exhibit a partial constitutive ethylene-response phenotype in the dark-grown seedling assay when grown in air (no ethylene), resulting in a shorter hypocotyl than the wild-type control (Fig. 3) [27]. Furthermore, the *etr1 etr2 ein4* mutant is hypersensitive to ethylene and exhibits a growth response to ethylene at concentrations that have no significant effect on the wild-type control (e.g. at less than 0.001 μL L^{-1} ethylene). The use of receptor loss-of-function mutants can therefore be used to evaluate the ability of transgenes to (1) rescue the constitutive ethylene-response phenotype of seedlings grown in air (no ethylene) and (2) mediate a seedling triple-response to ethylene [32].

As shown in Fig. 3, transgenic expression of either ETR1^{wt} or ETR1^{D25N} rescue the hypocotyl growth defect of the triple mutant *etr1 etr2 ein4* in air to a similar extent. Furthermore, the ethylene response of the dark-grown seedlings is also similar, although there are indications that the ETR1^{wt} lines are more sensitive to ethylene at some of the lower ethylene concentrations (e.g. 0.01 μL L^{-1} and below) (Fig. 3B-D). In contrast, when evaluated in the *etr1 etr2 ein4 ers1 ers2* background, substantial differences in ethylene sensitivity are observed for the ETR1^{wt} transgenic line compared to the ETR1^{D25N} transgenic lines (Fig. 3B-D; Fig. S1). The ETR1^{wt} line exhibits a slightly shorter hypocotyl than the ETR1^{D25N} lines in air, likely due to a heightened sensitivity to endogenous ethylene, and exhibits dramatic differences in its growth response at low ethylene concentrations. In particular, the ETR1^{wt} line exhibits a substantial decrease in growth to ethylene concentrations from 1×10^{-5} to 1×10^{-3} μL L^{-1}, concentrations that only minimally affect the response of the ETR1^{D25N} lines (Fig. 3D; Fig. S1). Interestingly, the amplitude of the ETR1^{wt} line growth response was similar across this 100-fold concentration range. Nevertheless, at higher ethylene concentrations (1 μL L^{-1} and above), both ETR1^{wt} or ETR1^{D25N} lines exhibit a similar and strong growth response to ethylene. Thus, both the ETR1^{wt} or ETR1^{D25N} lines can perceive and respond to ethylene, but the ETR1^{wt} line is much more responsive to the lower ethylene concentrations.

For the analysis of the seedling triple-response to ethylene, we typically include aminoethoxyvinylglycine (AVG) as a competitive inhibitor of ethylene biosynthesis in the

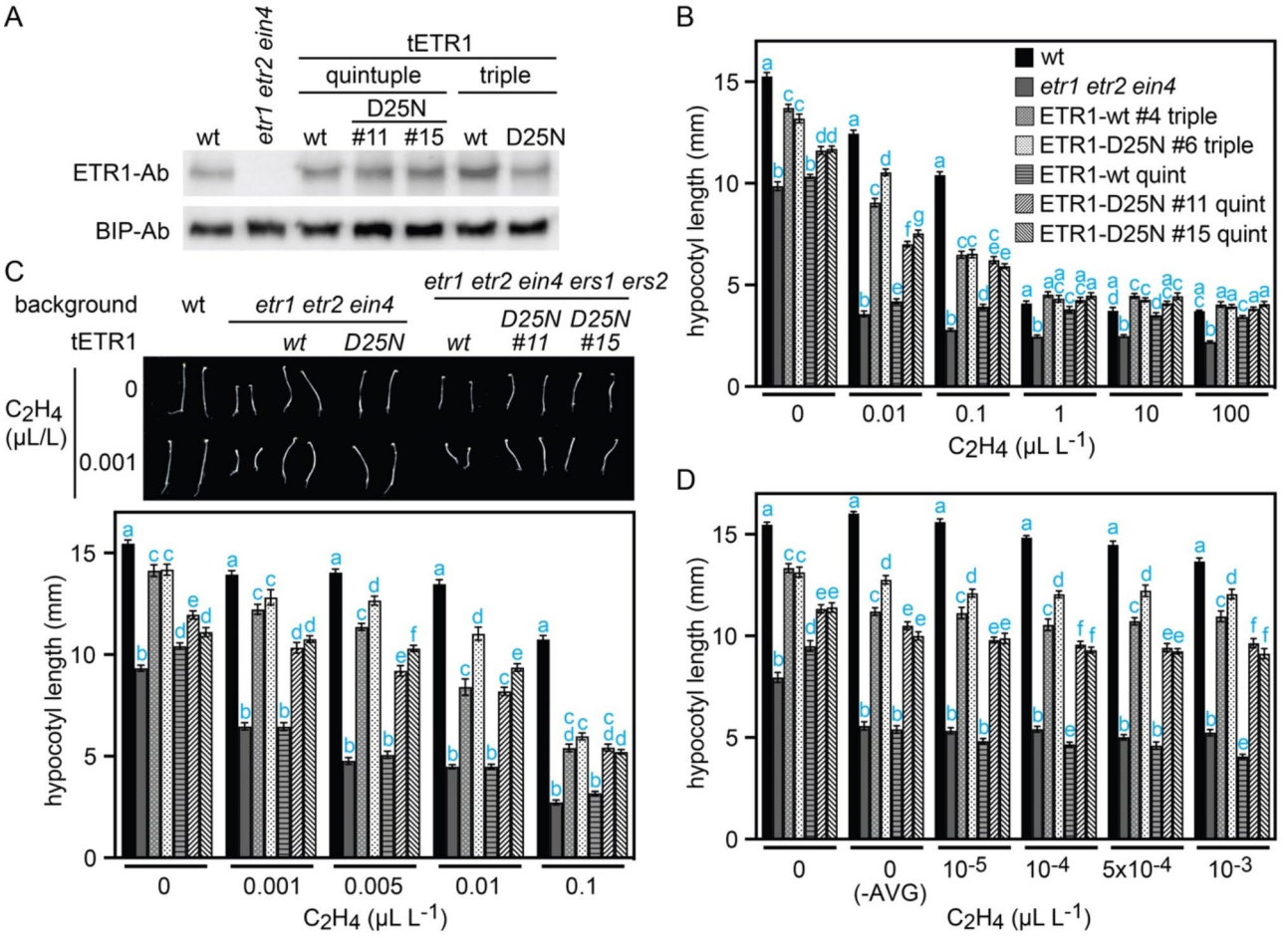

Fig. 3 Dark-grown seedling response to ethylene of tETR1^{wt} and tETR1^{D25N} lines. Wildtype and mutant versions of ETR1 were transgenically (tETR1) expressed in the *etr1 etr2 ein4* (triple mutant) and *etr1 etr2 ein4 ers1 ers2* (quintuple mutant) backgrounds. Wildtype and the *etr1 etr2 ein4* triple mutant serve as controls. The ethylene biosynthesis inhibitor AVG was incorporated into the growth media, unless otherwise indicated, to inhibit ethylene production by the seedlings. **(A)** ETR1 protein levels as determined by immunoblot analysis with an anti-ETR1 antibody for 4-day-old dark grown seedlings. BIP serves as a loading control. **(B-D)** Dose response to ethylene for hypocotyl growth of 4-day-old dark-grown seedlings. Representative seedlings are shown for the response to 0.001 µL L-1 ethylene in panel **C**. The effect of removing the ethylene biosynthesis inhibitor AVG from the growth media is shown in panel **D**. Different blue letters indicate a significant difference in hypocotyl length among the lines at each ethylene concentration based on ANOVA analysis with post hoc Holm multiple comparisons test ($n = 20$; $p < 0.05$). Error bars represent SE

growth media, so that the dose response of seedlings to exogenous ethylene is minimally affected by endogenous ethylene production. Furthermore, for growth of seedlings in the absence of ethylene (in air), hydrocarbon-free air is circulated through the growth chamber to prevent the accumulation of endogenous ethylene in the chamber. To determine the effect of endogenous ethylene biosynthesis on the seedling growth response, we analyzed hypocotyl growth in the absence of AVG. As shown in Fig. 3D, when grown in air, hypocotyl growth of the wild-type control is not reduced and the ETR1^{D25N} lines exhibit only a slight growth reduction in media lacking AVG, consistent with low levels of ethylene biosynthesis and accumulation. In contrast, the *etr1 etr2 ein4* triple mutant and the tETR1^{wt} (*etr1 etr2 ein4 ers1 ers2*) line both exhibit a sharp decrease in hypocotyl growth in media lacking AVG (Fig. 3D), this sensitivity to endogenous ethylene consistent with the heightened sensitivity we observed for these lines to exogenous ethylene.

Effect of ETR1 ethylene-binding affinity on the short-term kinetic growth response of seedlings to ethylene

Clear differences were observed for the long-term ethylene growth responses of the tETR1^{wt} (*etr1 etr2 ein4 ers1 ers2*) and tETR1^{D25N} (*etr1 etr2 ein4 ers1 ers2*) lines, and so we also analyzed their short-term growth response to ethylene. For this purpose, we performed a short-term kinetic analysis for the ability of ethylene to inhibit hypocotyl growth [3, 33], with seedlings grown in the presence of AVG to inhibit endogenous ethylene production. Seedlings were assessed for their growth response to 10 nL L^{-1} (0.01 µL L^{-1}) ethylene (Fig. 4) as we had observed

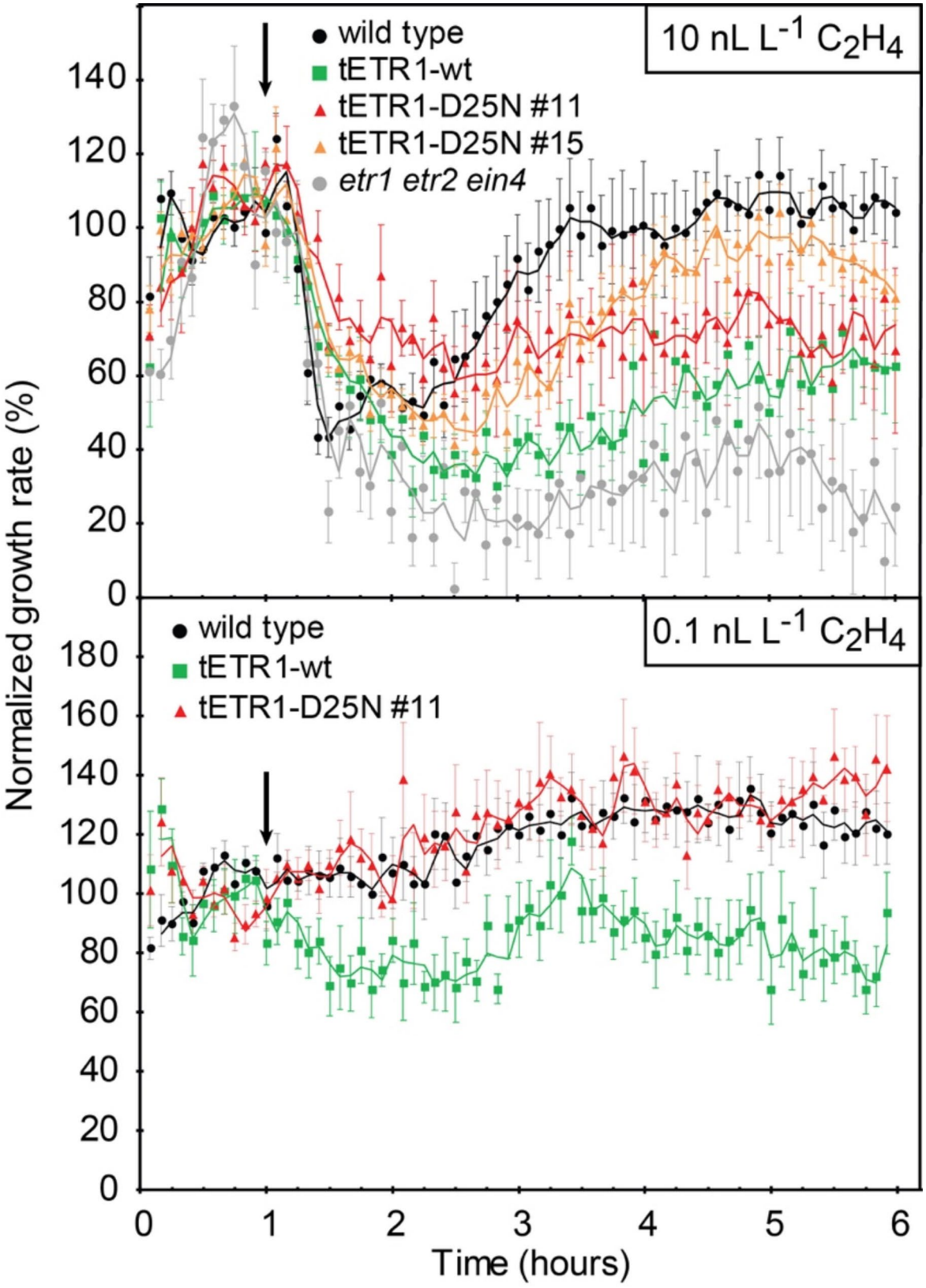

Fig. 4 Short-term kinetic growth response of dark-grown seedlings to ethylene. Wild type (wt), *etr1 etr2 ein4*, *tETR1*^{wt}, and *tETR1*^{D25N} lines #11 and #15 were examined at 10 nL L^{-1} and at 0.1 nL L^{-1} ethylene, as indicated. The ethylene biosynthesis inhibitor AVG was incorporated into the growth media to inhibit ethylene production by the seedlings. Basal growth rate prior to ethylene treatment was 0.35 (0.03), 0.17 (0.02), 0.20 (0.02), 0.19 (0.02), and 0.23 (0.02) mm hr^{-1} for wt, *etr1 etr2 ein4*, tETR1^{wt}, tETR1^{D25N} lines #11 and #15, respectively. Error bars represent SE ($n \geq 7$, 10 nL L^{-1} ethylene; $n \geq 5$, 0.1 nL L^{-1} ethylene)

significant differences among the lines in their long-term hypocotyl growth response to this ethylene concentration (Fig. 3B, C; Fig. S1). As shown in Fig. 4, the wild-type seedlings exhibit a transient growth response to this relatively low ethylene concentration, characterized by a rapid inhibition of the hypocotyl growth rate followed by recovery to the initial growth rate within 2.5 h after 10 nL L^{-1} ethylene application. This transient growth response to low ethylene concentrations for the wildtype is consistent with prior analysis [3]. The initial rapid response was found to be transcriptionally independent of the EIN3 and EIL1 transcription factors, with a weakly detectable response occurring at ethylene concentrations as low as 0.2 nL L^{-1}. The protracted ethylene response required the presence of EIN3 and EIL1, along with higher ethylene concentrations to be maintained.

The sensitized triple-mutant *etr1 etr2 ein4*, although exhibiting a rapid inhibition of growth in response to ethylene like the wild-type, maintains a low growth rate over the period of the kinetic analysis (Fig. 4). The $tETR1^{wt}$ (*etr1 etr2 ein4 ers1 ers2*) and $tETR1^{D25N}$ (*etr1 etr2 ein4 ers1 ers2*) lines exhibit a short-term kinetic response to ethylene intermediate between that of the wild type and *etr1 etr2 ein4*, characterized by a rapid inhibitory growth response to ethylene but only a partial recovery toward their initial growth rate (Fig. 4). Notably, $tETR1^{wt}$ line exhibited a stronger inhibitory growth response and slower recovery kinetics than the $ETR1^{D25N}$ lines, consistent with the $tETR1^{wt}$ line having a higher affinity for ethylene and so being able to mediate a more pronounced rapid-growth response.

We further compared the response of the $tETR1^{wt}$ (*etr1 etr2 ein4 ers1 ers2*) line to a $tETR1^{D25N}$ (*etr1 etr2 ein4 ers1 ers2*) line for the short-term kinetic response at the 100-fold lower ethylene concentration of 0.1 nL L^{-1} (1×10^{-4} µL L^{-1}). As shown in Fig. 4, the wild-type control exhibits no hypocotyl growth inhibition at this ethylene concentration, consistent with previous findings [3]. The $tETR1^{D25N}$ line also exhibits no growth inhibition in response to 0.1 nL L^{-1} ethylene, its kinetic response similar to that of the wild-type control (Fig. 4). In contrast, the $tETR1^{wt}$ line exhibits hypocotyl growth inhibition in response to 0.1 nL L^{-1} ethylene, although the kinetics for this response are not as rapid or pronounced as those seen at the higher ethylene concentration (Fig. 4). Taken together, analysis of the short-term kinetic response of the seedlings to ethylene is consistent with $ETR1^{wt}$ having a higher affinity for ethylene than $ETR1^{D25N}$ and therefore being competent to mediate physiological responses to ethylene at lower ethylene concentrations. Additionally, both the long-term and short-term analyses reveal that Arabidopsis seedlings have an ability to sense ethylene at lower concentrations than previously deduced, as found with both the *etr1 etr2 ein4* triple mutant and the $tETR1^{wt}$ (*etr1 etr2 ein4 ers1 ers2*) line.

Effect of ETR1 ethylene-binding affinity on the molecular response of seedlings to ethylene

As a complement to the physiological growth-response assays described above, we examined the molecular response of the lines based on ethylene-dependent gene expression. For this purpose, we examined the expression of genes that are rapidly induced or suppressed in response to exogenous ethylene treatment. Expression of *ERF1*, *ERS1*, and *OSR1* is induced by 1 µL L^{-1} ethylene, the promoter region of each gene containing binding sites for the EIN3/EIL family of transcription factors that mediate the primary response to ethylene [5, 25, 30, 34–36]. Expression of *EXPB1*, *EXP5*, and *CAPE2* is suppressed in response to 1 µL L^{-1} ethylene [30] and, based on ChIP-seq analysis, do not contain EIN3 binding sites in their promoter regions [36].

For the molecular analysis, four-day-old dark-grown seedlings were treated in the absence or presence of 1 and 0.01 µL L^{-1} exogenous ethylene for two hours and gene expression examined by qRT-PCR (Fig. 5). These ethylene concentrations were chosen based on the responsiveness of the $tETR1^{wt}$ (*etr1 etr2 ein4 ers1 ers2*) and $tETR1^{D25N}$ (*etr1 etr2 ein4 ers1 ers2*) lines when examined for their growth response to ethylene (Figs. 3 and 4; Fig. S1); the lines exhibited a similar growth response to 1 µL L^{-1} ethylene, but $tETR1^{D25N}$ line was significantly less responsive than $tETR1^{wt}$ lines to 0.01 µL L^{-1} ethylene. As shown in Fig. 5, the basal level of expression for the ethylene-induced genes is generally higher than in wild-type for the $tETR1^{wt}$ and $ETR1^{D25N}$ lines, whereas the basal expression level for ethylene-repressed genes is generally lower for the $tETR1^{wt}$ and $ETR1^{D25N}$ lines. This effect on basal gene expression is anticipated because loss of multiple ethylene-receptor family members, such as in the quintuple mutant background, results in a partial constitutive ethylene-response phenotype [26–28]. A similar effect on basal expression levels is seen in the *etr1 etr2 ein4* triple mutant (Fig. 5).

The effects of ethylene on gene expression are consistent with the physiological growth response to ethylene of the $tETR1^{wt}$ and $ETR1^{D25N}$ lines. All the lines are responsive to 1 µL L^{-1} ethylene, but the $ETR1^{D25N}$ lines are less responsive than the $tETR1^{wt}$ line at 0.01 µL L^{-1} ethylene (Fig. 5). Indeed, whereas the $tETR1^{wt}$ line exhibits significant differences in expression for the six reporter genes in response to ethylene, the $ETR1^{D25N}$ lines exhibit no significant difference in expression for five out of the six reporters (*OSR1*, *ERS1*, *EXP5*, *CAPE2*, *EXPb1*). We note that for the ethylene-induced genes, the basal level of expression in the $ETR1^{D25N}$ lines trends higher than that of the $tETR1^{wt}$ line, potentially

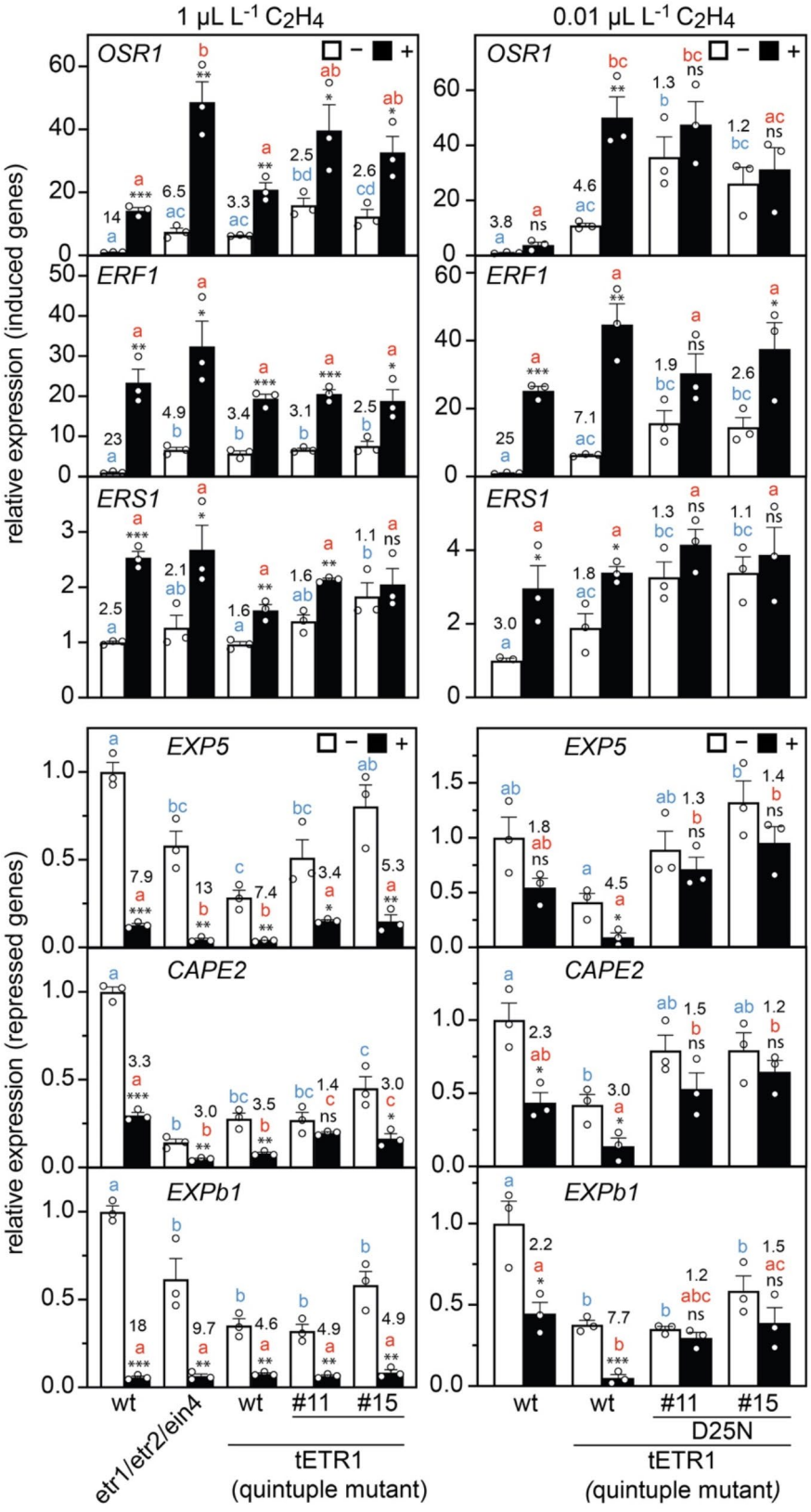

Fig. 5 (See legend on next page.)

(See figure on previous page.)

Fig. 5 Molecular response of dark-grown seedlings to ethylene. Wild type (wt), *etr1 etr2 ein4*, *tETR1*^{wt}, and *tETR1*^{D25N} lines #11 and #15 were examined. Seedlings were treated for 2 h with 1 µL/L or 0.01 µL/L ethylene. Ethylene-induced genes (*OSR1*, *ERF1*, and *ERS1*) and ethylene-repressed genes (*EXP5*, *CAPE2*, and *EXPb1*) were examined. Expression is relative to the untreated wild type (wt, - C_2H_4). The t-test was used for each line to assess significant changes in gene expression in response to ethylene (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns, not significant). Different blue (-ethylene) or red (+ethylene) letters indicate a significant difference in expression among the lines based on ANOVA analysis with post hoc Holm multiple comparisons test ($n = 20$; $p < 0.05$). Numbers indicate fold-change in response to ethylene (+ C_2H_4/- C_2H_4 for induced genes; - C_2H_4/+ C_2H_4 for repressed genes). Error bars represent SE

complicating the interpretation for the differences in gene expression. However, differences in sensitivity for tETR1^{wt} and ETR1^{D25N} lines are clear when considering those genes whose expression is suppressed in response to ethylene. As such, based on gene expression analysis at 0.01 µL L^{-1} ethylene, the tETR1^{D25N} (*etr1 etr2 ein4 ers1 ers2*) lines are hyposensitive compared to the tETR1^{wt} (*etr1 etr2 ein4 ers1 ers2*) line.

Discussion

Ethylene receptors bind their ligand ethylene with high affinity. For example, the ethylene receptor ETR1 of Arabidopsis binds ethylene with a calculated dissociation constant (K_d) of 0.036 µL L^{-1} (2.4 nM), and has a half-life for dissociation of 12.5 h [7]. The ethylene affinity of the receptors is so high that release of ethylene occurs too slowly to facilitate the receptors being reused for signaling, and the receptors are instead degraded following ethylene binding through a proteasome-dependent pathway [13, 37, 38]. We previously determined that a site-directed mutation of Asp25 to Asn in ETR1 (ETR1^{D25N}) was a functional receptor, but with at least 100-fold less affinity for ethylene than the wild-type ETR1 receptor (ETR1^{wt}) [30]. Here, in the current study, we examined the consequences of such changes in ethylene binding affinity upon plant growth and development.

By making use of an Arabidopsis background functionally lacking all five native receptors (*etr1 etr2 ers1 ers2 ein4*), we were able to assess the relative activity of transgenically expressed ETR1^{wt} and ETR1^{D25N} using physiological and molecular assays. From this analysis, we determined that changes in ethylene binding affinity of ETR1 are reflected in plant growth and responses to ethylene. These differences between ETR1^{wt} and ETR1^{D25N} lines occur in seedlings grown under light or dark conditions, and when assayed for short- or long-term responses to ethylene. The dose response to ethylene in dark-grown seedlings is particularly revealing as to differences conferred by expression of ETR1^{wt} and ETR1^{D25N}. Expression of tETR1^{wt} exhibited a long-term dark-grown seedling response at the lowest ethylene concentration examined (1×10^{-5} µL L^{-1} or 0.01 nL L^{-1} ethylene), and with the sensitivity of its response confirmed in the short-term kinetics growth response assay at 0.1 nL L^{-1} ethylene. This result contrasts with the tETR1^{D25N} lines, which exhibited minimal growth response to ethylene concentrations at 0.001 µL L^{-1} (1 nL L^{-1}) or lower in the long-term dark-grown seedling assay, and were indistinguishable from the wild-type control in the short-term kinetics growth response assay at 0.1 nL L^{-1} ethylene. Based on the dose-response analysis, there is at least a 100-fold difference in ethylene sensitivity when comparing plants expressing tETR1^{wt} to tETR1^{D25N}. This difference in plant sensitivity is consistent with the 100-fold or greater difference in ethylene binding affinity of the ETR1^{wt} and ETR1^{D25N} receptors, based on a [^{14}C]ethylene binding assay using a heterologous yeast expression system [30].

The finding that the ethylene affinity of ETR1 can be perturbed by the single site-directed mutation Asp25Asn raises the question as to whether other mutations might similarly affect ethylene sensitivity. When considering this question, it is important to contextualize the role that Asp25 plays in the mechanism for ethylene signaling in ETR1. Based on molecular modeling and genetic analysis, Asp25 performs a bifunctional role [30]. First, Asp25 forms a polar bond to a critical His residue (His69) important for chelating the copper cofactor that binds ethylene [30]. Second, Asp25 forms another polar bond to Lys91 in the third transmembrane segment implicated in transmitting conformational changes induced by ethylene binding [30, 39]. Mutation of Asp25 to Asn eliminates the polar bond to His69, thereby weakening the copper metal complexation and indirectly reducing the affinity of the receptor for ethylene [30]. However, mutation of Asp25 to Asn still preserves the polar bond to Lys91 and the ability to transmit conformational changes to the third transmembrane segment [30].

Based on the specific role of Asp25 in ETR1, it is unlikely that another residue would function similarly in the ethylene receptors. However, mutation of other residues of ETR1 could potentially affect the kinetics for ethylene binding by perturbing the binding site or restricting the ability of ethylene to reach the binding site. Of relevance to such possibilities, prior mutagenesis studies have evaluated multiple residues in the transmembrane domain of ETR1 for their effect on ethylene binding and signaling [7, 11, 12, 39]. From these studies, 37 residues associated with the transmembrane domain of ETR1 were evaluated, most residues replaced with Ala, others with more conserved substitutions or with mutations originally found to confer ethylene insensitivity through forward genetic analysis. From these analyses, there is a strong correlation between those mutations that

eliminated or severely reduced ethylene binding in the transgenic yeast assay and an ethylene-insensitive physiological growth response. This includes the Asp25Ala mutation [39], which we independently confirmed has reduced binding and confers ethylene insensitivity in planta, differing in the later effect from what we found with the Asp25Asn mutation.

Interestingly, some site-directed mutations exhibit an apparent reduction in ethylene binding but still confer a physiological growth response to ethylene. These include Phe33 and Ser34 in the first transmembrane segment, His107 in the third transmembrane segment, and Pro110 following the third transmembrane segment [39]. It remains to be seen if the decrease in ethylene binding is due to a change in binding kinetics as we found for the $ETR1^{D25N}$ mutation. If owed to altered ethylene binding kinetics (~55% ethylene binding for $ETR1^{S34A}$; ~40% binding for $ETR1^{H107A}$ and $ETR1^{P110A}$; ~15% binding for $ETR1^{F33A}$ when compared to $ETR1^{wt}$) [39], the change is much less dramatic than that found with the $ETR1^{D25N}$ mutation (100-fold or greater decrease in ethylene binding compared to $ETR1^{wt}$) [30]. Furthermore, the current structural model for the ETR1 dimer suggests that these residues are exposed on the surface of the receptor and do not directly impact the internal region involved in ethylene binding [30]; as such, the decrease in [^{14}C]ethylene binding may arise from a decrease in protein expression when using the heterologous yeast system and not be directly related to ethylene binding kinetics. Thus, aside from $ETR1^{D25N}$, none of the mutations made within the ethylene binding domain of ETR1 appear to substantially affect ethylene binding kinetics while still retaining receptor functionality. Nevertheless, the phenotypic differences we observe for various mutations at a single amino-acid residue ($ETR1^{D25A}$ compared to $ETR1^{D25N}$) point to how, based on the structure of the ethylene binding site, other targeted mutations can potentially be used modulate ethylene binding kinetics.

In addition to lending insight into the role that ethylene binding kinetics plays in the plant response to ethylene, our results also demonstrate that plants have greater sensitivity to ethylene than previously recognized. It was previously found that wild-type seedlings are capable of weakly responding to 0.2 nL L^{-1} ethylene, based on short-term kinetic growth analysis [3]. We found that both $tETR1^{wt}$ (*etr1 etr2 ers1 ers2 ein4*) and the *etr1 etr2 ein4* triple mutant exhibit a long-term dark-grown seedling response at the lowest ethylene concentration examined (0.01 nL L^{-1} ethylene). Furthermore, the enhanced sensitivity of the $tETR1^{wt}$ (*etr1 etr2 ers1 ers2 ein4*) line was confirmed in the short-term kinetics growth response assay at 0.1 nL L^{-1} ethylene. A common feature of both lines is that they only express a subset of the receptors, solely *tETR1^{wt}* in the *etr1 etr2 ers1 ers2 ein4* background, and *ERS1* and *ERS2* in the *etr1 etr2 ein4* background. These can be considered sensitized backgrounds because ethylene receptors function as negative regulators of ethylene signaling, such that they suppress ethylene responses in the absence of ethylene (in air) and, upon binding ethylene, are inactivated to relieve the suppression [8, 9, 26–28]. Loss of receptors can therefore result in ethylene hypersensitivity and/or a constitutive ethylene-response phenotype [8, 9, 26–28, 40]. However, it was not previously recognized that such lines would be capable of detecting and responding to such low levels of ethylene as examined here. Furthermore, based on our results using different mutant backgrounds, this ability to sense and respond to such low ethylene concentrations can be mediated by multiple members of the receptor family. Based on these results, one would predict that a subset of the ethylene receptors would bind ethylene at similarly low concentrations in wild-type seedlings, but it is unclear whether this would be sufficient to confer a physiological or molecular response.

Short-term kinetic analysis has revealed two phases to the growth inhibition of dark-grown seedlings to ethylene [3, 33]. The first phase is rapid and independent of the EIN3 and EIL1 transcriptional activity. The second phase is delayed and EIN3/EIL1-dependent. The first phase of growth inhibition is more sensitive to ethylene than the second phase, such that wild-type seedlings exposed to ethylene concentrations ≤ 10 nL L^{-1} exhibit a transient first-phase response before recovering to pretreatment growth rates even though in the continued presence of ethylene. Our kinetic analysis revealed that $tETR1^{wt}$ (*etr1 etr2 ers1 ers2 ein4*) and the *etr1 etr2 ein4* triple mutant do not fully recover to pretreatment growth rates in the presence of ethylene concentrations ≤ 10 nL L^{-1}. This likely facilitated our ability to detect heightened sensitivity to low concentrations of ethylene in the long-term growth response assay. Interestingly, the long-term growth response assay for these two lines also indicated that the amplitude of their growth response did not appreciably vary from 0.00001 to 0.001 µL L^{-1} ethylene (0.01 to 1 nL L^{-1}). It was previously determined with wild-type seedlings that, when fully engaged (ethylene concentrations above 1 nL L^{-1}), the amplitude of the first phase growth response is the same [3]. The similarity in amplitude we find for the ethylene response at low ethylene concentrations, is consistent with this response being mediated through the first-phase mechanism, and suggests that this response may be maintained for a longer duration than previously known.

How the receptors can mediate a response to such low ethylene concentrations remains an unresolved question. Based on our analyses, the receptors can mediate a response to ethylene at concentrations at least 1000-fold lower than the K_d of the receptors for ethylene [7,

41], which suggests that mechanisms exist to amplify the signal because so few receptors would bind ethylene at the low concentrations. Mechanisms involving magnitude amplification and sensitivity amplification, based on examples from other signaling systems, have been proposed to explain the sensitivity of the ethylene response [5]. Magnitude amplification occurs when downstream elements in a signaling cascade, typically enzymes, regulate multiple substrates when activated [42]. In the case of the ethylene signaling cascade, the CTR1 Ser/Thr kinase [43–46] and the EIN3/EIL family of transcription factors [35, 47] represent points at which signals emanating from the receptors could be amplified. Sensitivity amplification occurs when a signal elicits a greater response than predicted from Michaelis-Menten kinetics [42]. Based on signaling by the evolutionarily related chemoreceptors of bacteria, it has been proposed that conformational changes may be transmitted among ethylene receptor clusters, such that "empty" receptors take on the conformation of a neighboring ethylene-bound receptor [3, 48]. Alternatively, ethylene released from one receptor could bind to another proximate receptor, in this manner effectively increasing the local ethylene concentration. Consistent with these possibilities for sensitivity amplification is the finding that ethylene receptors form higher order clusters [16, 49, 50].

Our results demonstrate that functional ethylene receptors can be generated that exhibit substantial differences in their ethylene binding affinities, and that such differences are reflected in a plant's physiological and molecular responses to ethylene. Such differences in ethylene affinity can serve both natural and commercial purposes. Naturally, although most ethylene receptors preserve Asp25 as a critical element of their ethylene binding domain, a small percentage of receptors are variants in which Asn is found at the Asp25 position, these variants thus being more sensitive to changes in ethylene concentration, since they would rapidly release ethylene once bound [30]. These Asn-variants are found in multiple genera of cyanobacteria (e.g. Kaiparowitsia, Leptolyngbya, Stenomitos, Thermoleptolyngbya, Trichocoleus, and Tildeniella). In cyanobacteria, the more responsive kinetics for ethylene binding is likely to facilitate chemotaxis [51] and/or changes in phototaxis mediated by ethylene [52]. Such Asn-variants would have lower ethylene binding affinity but, as noted above, mechanisms exist by which bacterial chemoreceptors can amplify there sensitivity to low ligand concentrations. Commercially, a receptor that is more responsive to changes in the environmental ethylene concentration should facilitate the development of ethylene nanosensors for agricultural and industrial use [53, 54]. The potential for improving ethylene nanosensors arises not only from a better understanding of the mechanism for ethylene binding, but also because the ethylene receptors themselves could potentially be adapted for use as biosensors. Membrane protein-based biosensors are attractive in comparison to human-made sensors because of their sensitivity and specificity for ligand detection [55]. Whereas $ETR1^{wt}$ could potentially be used for sensitive detection of ethylene, its tight binding to ethylene would not allow for real-time detection of changes in ethylene levels. In contrast, the rapid release kinetics of $ETR1^{D25N}$ for ethylene would facilitate the development of biosensors capable of following real-time changes in ethylene levels.

Materials and methods

Plant materials and growth conditions

All Arabidopsis lines used in this study were of the Columbia (Col-0) accession. The *etr1-6 etr2-3 ein4-4* triple mutant has been described [27]. Generation of the following transgenic lines involving $ETR1^{wt}$ and the mutant $ETR1^{D25N}$ in the triple-mutant *etr1 etr2 ein4* and the quintuple-mutant *etr1 etr2 ein4 ers1 ers2* backgrounds has been described [30]: $tETR1^{wt}$-#4 (*etr1 etr2 ein4*), $tETR1^{D25N}$-#6 (*etr1 etr2 ein4*), $tETR1^{D25N}$-#11 (*etr1 etr2 ein4 ers1 ers2*), and $tETR1^{D25N}$-#15 (*etr1 etr2 ein4 ers1 ers2*). For analysis of adult phenotypes, seedlings were initially grown on Murashige and Skoog basal medium with MES buffer (pH 5.7) and vitamins (Research Products International), 1% (w/v) sucrose, and 0.8% (w/v) phytoagar (Research Products International) under constant light (100 μmol m^{-2} s^{-1}) at 22 °C. Green seedlings were then transferred to soil at one plant per pot, and grown at 22 °C under long-day conditions (16 h day/8 hr night) with light intensity of 100 μmol m^{-2} s^{-1} [56]. The triple response of dark-grown seedlings to ethylene was performed as described, in the presence of 5 μM AVG to inhibit ethylene biosynthesis, and hypocotyl measurements of the seedlings performed after four days growth [25]. The short-term kinetic analysis of dark-grown seedlings was performed as described in the presence of 5 μM AVG [3, 33].

Generation of CRISPR-Cas9 mutant lines targeting *ERS1* and *ERS2*

The CRISPR cassette targeting ERS1 and ERS2 has been previously described [30]. To generate a $tETR1^{wt}$ transgene in the quintuple-mutant *etr1 etr2 ein4 ers1 ers2* background, the CRISPR-ERS1/ERS2 plasmid was introduced into the $tETR1^{wt}$-#4 (*etr1 etr2 ein4*) line by the floral dip method [57]. Genomic DNA was isolated [58] and the region surrounding the CRISPR target sequence amplified by PCR and sequenced as described [30]. The presence of the T-DNA insert containing the Cas9 cassette was determined as described [30]. Through this approach, an indel mutation in *ERS2* was identified containing a six-base-pair deletion: GCCTTCACCT

T(acaatt)GGGTGGTCT (deleted nucleotides shown in lower case); however, no mutations in *ERS1* were identified. We therefore crossed a Cas9(-/-) plant of this tETR1^{wt} line to tETR1^{D25N}-#11 (*etr1 etr2 ein4 ers1 ers2*), and in the subsequent segregating generations identified tETR1^{wt} (*etr1 etr2 ein4 ers1 ers2*), in which both the *ers1* (1-bp insertion of T) and *ers2* (1-bp deletion of T) alleles were derived from the tETR1^{D25N}-#11 (*etr1 etr2 ein4 ers1 ers2*) line (Fig. 1B). Primers used for the analysis of the CRISPR-Cas9-derived lines are listed in Table S1.

Immunoblot analysis

Immunoblot analysis was performed with the microsomal fraction of seedlings as described [37]. ETR1 was identified with a polyclonal anti-ETR1 antibody, and BIP identified with an anti-BIP antibody to serve as a loading control [14]. The chemiDoc MP imaging system (BIO-RAD) was used to visualize chemiluminescence of immunodecorated proteins.

RNA expression analysis

RNA isolation was performed with the RNeasy Plant Mini Kit (Qiagen, USA) and cDNA synthesized using the First Strand cDNA Synthesis Kit (Invitrogen, USA). RT-qPCR was performed with three biological replicates and two technical replicates, using the *beta-tubulin* gene (At5g62700) for normalization. Primers for genes are listed in Table S1.

Statistical analysis

Unpaired T-tests were performed in Prism (GraphPad Software, Inc.). ANOVA-based statistical analyses were performed using an online calculator (astatsa.com/).

Gene identifiers

ETR1 (At1g66340), *ETR2* (At3g23150), *EIN4* (At3g04580), *ERS1* (At2g40940), *ERS2* (At1g04310), *ERF1* (At3g23240), *OSR1* (At2g41230), *EXP5* (At3g29030), *EXPb1* (At2g20750), *CAPE2* (At4G25780), *B-TUB3* (At5G62700).

Abbreviations

AVG	Aminoethoxyglycine
D	Asn
N	Asn

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-025-06469-y.

Supplementary Material 1

Supplementary Material 2

Acknowledgements

Not applicable.

Author contributions

GES conceived the project. SA1, GES, and BMB designed research. SA1, SA2, ED, SS, SNS, and BMB performed research. SA1, SA2, ED, GES, SNS, and BMB analyzed data. SA1, GES, and BMB were involved in visualization. GES, SNS, and NL were involved in project administration and supervision. SA1 and GES wrote the original draft. SA1, SA2, ED, SS, NL, SNS, BMB, and GES reviewed and edited the manuscript.

Funding

This work was supported by National Science Foundation grants IOS-1856513 and IOS-2425472 to G.E.S, MCB-1817304 and MCB-1716279 to B.M.B., and MCB-1517032 to G.E.S and B.M.B.; and grants 16103615, 16100318, 16101819, 16101920, 16306919 from the RGC of Hong Kong to N.L.

Data availability

Data generated or analyzed during this study are included in this published article or are available from the corresponding author on reasonable request. Plant materials are available through The Arabidopsis Biological Resource Center (ABRC) or from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

A patent application related to this work has been filed (application #18/907,725. Ethylene receptors and binding domains with modified binding kinetics for ethylene).

Received: 29 December 2024 / Accepted: 26 March 2025
Published online: 05 April 2025

References

1. Mattoo AK, Suttle JC, editors. The plant hormone ethylene. Boca Raton: CRC Press, Inc.; 1991.
2. Abeles FB, Morgan PW, Saltveit ME Jr. Ethylene in plant biology. 2nd ed. San Diego: Academic; 1992.
3. Binder BM, Mortimore LA, Stepanova AN, Ecker JR, Bleecker AB. Short-term growth responses to ethylene in Arabidopsis seedlings are EIN3/EIL1 independent. Plant Physiol. 2004;136:2921–7.
4. Chen QG, Bleecker AB. Analysis of ethylene signal transduction kinetics associated with seedling-growth responses and chitinase induction in wild-type and mutant Arabidopsis. Plant Physiol. 1995;108:597–607.
5. Azhar BJ, Zulfiqar A, Shakeel SN, Schaller GE. Amplification and adaptation in the ethylene signaling pathway. Small Methods 2019:1900452.
6. Chang C, Kwok SF, Bleecker AB, Meyerowitz EM. Arabidopsis ethylene response gene *ETR1*: similarity of product to two-component regulators. Science. 1993;262:539–44.
7. Schaller GE, Bleecker AB. Ethylene-binding sites generated in yeast expressing the Arabidopsis *ETR1* gene. Science. 1995;270:1809–11.
8. Binder BM, Chang C, Schaller GE. Perception of ethylene by plants: Ethylene receptors. In: Annual Plant Reviews: The Plant Hormone Ethylene. Edited by McManus MT, vol. 44: Wiley-Blackwell; 2012:117–145.
9. Binder BM. Ethylene signaling in plants. J Biol Chem. 2020;295:7710–25.
10. Ma Q, Dong C-H. Regulatory functions and molecular mechanisms of ethylene receptors and receptor-associated proteins in higher plants. Plant Growth Reg. 2021;93:39–52.
11. Rodriguez FI, Esch JJ, Hall AE, Binder BM, Schaller GE, Bleecker AB. A copper cofactor for the ethylene receptor ETR1 from Arabidopsis. Science. 1999;283:996–8.
12. Hall AE, Chen QG, Findell JL, Schaller GE, Bleecker AB. The relationship between ethylene binding and dominant insensitivity conferred by mutant forms of the ETR1 ethylene receptor. Plant Physiol. 1999;121:291–9.
13. Chen Y-F, Shakeel SN, Bowers J, Zhao X-C, Etheridge N, Schaller GE. Ligand-induced degradation of the ethylene receptor ETR2 through

a proteasome-dependent pathway in Arabidopsis. J Biol Chem. 2007;282:24752–8.
14. Chen YF, Randlett MD, Findell JL, Schaller GE. Localization of the ethylene receptor ETR1 to the Endoplasmic reticulum of Arabidopsis. J Biol Chem. 2002;277:19861–6.
15. Dong C-H, Rivarola M, Resnick JS, Maggin BD, Chang C. Subcellular co-localization of Arabidopsis RTE1 and ETR1 supports a regulatory role for RTE1 in ETR1 ethylene signaling. Plant J. 2008;53:275–86.
16. Grefen C, Städele K, Růžička K, Obrdlik P, Harter K, Horák J. Subcellular localization and in vivo interaction of the *Arabidopsis Thaliana* ethylene receptor family members. Mol Plant. 2008;1:308–20.
17. Beyer J. Silver ion, a potent inhibitor of ethylene action in plants. Plant Physiol. 1976;58:268–71.
18. Burg SP, Burg EA. Molecular requirements for the biological activity of ethylene. Plant Physiol. 1967;42:144–52.
19. Binder BM, Rodriguez FI, Bleecker AB, Patterson SE. The effects of group 11 transition metals, including gold, on ethylene binding to the ETR1 receptor and growth of Arabidopsis Thaliana. FEBS Lett. 2007;581:5105–9.
20. Schott-Verdugo S, Muller L, Classen E, Gohlke H, Groth G. Structural model of the ETR1 ethylene receptor transmembrane sensor domain. Sci Rep. 2019;9:8869.
21. Cutsail G 3rd, Schott-Verdugo S, Muller L, DeBeer S, Groth G, Gohlke H. Spectroscopic and QM/MM studies of the Cu(I) binding site of the plant ethylene receptor ETR1. Biophys J. 2022;121:3862–73.
22. Schaller GE, Kieber JJ. Ethylene. The Arabidopsis Book (C Somerville, E Meyerowitz, editors) 2002:1–18.
23. Gamble RL, Coonfield ML, Schaller GE. Histidine kinase activity of the ETR1 ethylene receptor from Arabidopsis. Proc Natl Acad Sci USA. 1998;95:7825–9.
24. Schaller GE, Shiu SH, Armitage JP. Two-component systems and their co-option for eukaryotic signal transduction. Curr Biol. 2011;21:R320–330.
25. Hall BP, Shakeel SN, Amir M, Haq NU, Qu X, Schaller GE. Histidine kinase activity of the ethylene receptor ETR1 facilitates the ethylene response in Arabidopsis. Plant Physiol. 2012;159:682–95.
26. Wang W, Hall AE, O'Malley R, Bleecker AB. 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. 2003;100:352–7.
27. Hua J, Meyerowitz EM. Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis Thaliana*. Cell. 1998;94:261–71.
28. Qu X, Hall BP, Gao Z, Schaller GE. A strong constitutive ethylene response phenotype conferred on Arabidopsis plants containing null mutations in the ethylene receptors *ETR1* and *ERS1*. BMC Plant Biol. 2007;7:3.
29. Bleecker AB, Estelle MA, Somerville C, Kende H. Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis Thaliana*. Science. 1988;241:1086–9.
30. Azhar BJ, Abbas S, Aman S, Yamburenko MV, Chen W, Muller L, Uzun B, Jewell DA, Dong J, Shakeel SN, et al. Basis for high-affinity ethylene binding by the ethylene receptor ETR1 of Arabidopsis. Proc Natl Acad Sci U S A. 2023;120:e2215195120.
31. Guzmán P, Ecker JR. Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. Plant Cell. 1990;2:513–23.
32. Qu X, Schaller GE. Requirement of the histidine kinase domain for signal transduction by the ethylene receptor ETR1. Plant Physiol. 2004;136:2961–70.
33. Binder BM, O'Malley RC, Wang W, Moore JM, Parks BM, Spalding EP, Bleecker AB. Arabidopsis seedling growth response and recovery to ethylene. A kinetic analysis. Plant Physiol. 2004;136:2913–20.
34. Solano R, Stepanova A, Chao Q, Ecker JR. Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. Genes Dev. 1998;12:3703–14.
35. Chao Q, Rothenberg M, Solano R, Roman G, Terzaghi W, Ecker JR. Activation of the ethylene gas response pathway in Arabidopsis by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. Cell. 1997;89:1133–44.
36. Chang KN, Zhong S, Weirauch MT, Hon G, Pelizzola M, Li H, Huang SS, Schmitz RJ, Urich MA, Kuo D, et al. Temporal transcriptional response to ethylene gas drives growth hormone cross-regulation in Arabidopsis. eLife. 2013;2:e00675.
37. Shakeel SN, Gao Z, Amir M, Chen YF, Rai MI, Haq NU, Schaller GE. Ethylene regulates levels of ethylene Receptor/CTR1 signaling complexes in Arabidopsis Thaliana. J Biol Chem. 2015;290:12415–24.
38. Kevany BM, Tieman DM, Taylor MG, Cin VD, Klee HJ. Ethylene receptor degradation controls the timing of ripening in tomato fruit. Plant J. 2007;51:458–67.
39. Wang W, Esch JJ, Shiu S-H, Agula H, Binder BM, Chang C, Patterson SE, Bleecker AB. Identification of important regions for ethylene binding and signaling in the transmembrane domain of the ETR1 ethylene receptor of Arabidopsis. Plant Cell. 2006;18:3429–42.
40. Cancel JD, Larsen PB. Loss-of-function mutations in the ethylene receptor ETR1 cause enhanced sensitivity and exaggerated response to ethylene in Arabidopsis. Plant Physiol. 2002;129:1557–67.
41. O'Malley RC, Rodriguez FI, Esch JJ, Binder BM, O'Donnell P, Klee HJ, Bleecker AB. Ethylene-binding activity, gene expression levels, and receptor system output for ethylene receptor family members from Arabidopsis and tomato. Plant J. 2005;41:651–9.
42. Koshland DE Jr., Goldbeter A, Stock JB. Amplification and adaptation in regulatory and sensory systems. Science. 1982;217:220–5.
43. Gao Z, Chen YF, Randlett MD, Zhao XC, Findell JL, Kieber JJ, Schaller GE. Localization of the Raf-like kinase CTR1 to the Endoplasmic reticulum of Arabidopsis through participation in ethylene receptor signaling complexes. J Biol Chem. 2003;278:34725–32.
44. Ju C, Yoon GM, Shemansky JM, Lin DY, Ying ZI, Chang J, Garrett WM, Kessenbrock M, Groth G, Tucker ML, et al. CTR1 phosphorylates the central regulator EIN2 to control ethylene hormone signaling from the ER membrane to the nucleus in Arabidopsis. Proc Natl Acad Sci U S A. 2012;109:19486–91.
45. Qiao H, Shen Z, Huang SS, Schmitz RJ, Urich MA, Briggs SP, Ecker JR. Processing and subcellular trafficking of ER-tethered EIN2 control response to ethylene gas. Science. 2012;338:390–3.
46. Clark KL, Larsen PB, Wang X, Chang C. Association of the Arabidopsis CTR1 Raf-like kinase with the ETR1 and ERS1 ethylene receptors. Proc Natl Acad Sci USA. 1998;95:5401–6.
47. Alonso JM, Stepanova AN, Solano R, Wisman E, Ferrari S, Ausubel FM, Ecker JR. 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. 2003;100:2992–7.
48. Binder BM. Rapid kinetic analysis of ethylene growth responses in seedlings: new insights into ethylene signal transduction. J Plant Growth Reg. 2007;26:131–42.
49. Gao Z, Schaller GE. The role of receptor interactions in regulating ethylene signal transduction. Plant Signal Behav. 2009;4:1152–3.
50. Gao Z, Wen C-K, Binder BM, Chen Y-F, Chang J, Chiang Y-H, Kerris RJ III, Chang C, Schaller GE. Heteromeric interactions among ethylene receptors mediate signaling in Arabidopsis. J Biol Chem. 2008;283:23081–810.
51. Mutalipassi M, Riccio G, Mazzella V, Galasso C, Somma E, Chiarore A, de Pascale D, Zupo V. Symbioses of cyanobacteria in marine environments: ecological insights and biotechnological perspectives. Mar Drugs 2021, 19.
52. Lacey RF, Binder BM. Ethylene regulates the physiology of the Cyanobacterium synechocystis Sp. PCC 6803 via an ethylene receptor. Plant Physiol. 2016;171:2798–809.
53. Vong K, Eda S, Kadota Y, Nasibullin I, Wakatake T, Yokoshima S, Shirasu K, Tanaka K. An artificial metalloenzyme biosensor can detect ethylene gas in fruits and Arabidopsis leaves. Nat Commun. 2019;10:5746.
54. Esser B, Schnorr JM, Swager TM. Selective detection of ethylene gas using carbon nanotube-based devices: utility in determination of fruit ripeness. Angew Chem Int Ed Engl. 2012;51:5752–6.
55. Misawa N, Osaki T, Takeuchi S. Membrane protein-based biosensors. J R Soc Interface 2018, 15.
56. Zubo YO, Blakley IC, Franco-Zorrilla JM, Yamburenko MV, Solano R, Kieber JJ, Loraine AE, Schaller GE. Coordination of Chloroplast development through the action of the GNC and GLK transcription factor families. Plant Physiol. 2018;178:130–47.
57. Clough SJ, Bent AF. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis Thaliana. Plant J. 1998;16:735–43.
58. Kasajima I, Ide Y, Ohkama-Ohtsu N, Yoneyama T, Fujiwara T. A protocol for rapid DNA extraction from *Arabidopsis Thaliana* for PCR analysis. Plant Mol Biol Rep. 2004;22:49–52.

Publisher's note