

ETHYLENE RESPONSE 1 Histidine Kinase Activity of Arabidopsis Promotes Plant Growth^{[C][W]}

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ETHYLENE RESPONSE 1 (ETR1) is one of five receptors for the plant hormone ethylene (Bleecker et al., 1988; Chang et al., 1993; for review, see Bleecker and Kende, 2000; Schaller and Kieber, 2002). ETR1 contains both an N-terminal ligand-binding hydrophobic domain and light signaling-implicated GAF domain (Fig. 1A; Chang et al., 1993). ETR1 also has a C-terminal His kinase (HK) domain fused with a response regulator motif.

Biochemical analyses have demonstrated functional HK activity (Gamble et al., 1998, 2002; Moussatche and Klee, 2004). Within the HK domain, both the catalytic G1 and ATP-binding G2 domains are required for the autophosphorylation of ETR1 at His-353. Protein-protein interaction between ETR1 and any of three Arabidopsis (*Arabidopsis thaliana*) His phosphotransfer proteins suggests that ETR1 HK activity may modulate Arabidopsis response regulators (ARRs) through two-component phosphor-relay system (Urao et al., 2000). Indeed, ETR1-dependent phosphorylation of the B-type ARR2 activates ethylene response transcription (Hass et al., 2004). Nevertheless, signaling through ARRs modulates ethylene signaling marginally, because combinations of B-type *arr* mutants are still sensitive to ethylene (Mason et al., 2005). Furthermore, ETR1 with inactive HK provides wild-type ethylene responsiveness in single *etr1* or double *etr1 ers1* null mutants (Gamble et al., 2002; Wang et al., 2003).

Thus far, the biological function or an in vivo signaling mechanism for ETR1 HK activity remains unknown. Only recently, the HK activity was shown to be important in the rate of growth recovery after ethylene removal (Binder et al., 2004). Otherwise, its role in ethylene or any other plant signaling is unclear.

To examine ETR1 HK function in vivo, we generated transgenic lines of *etr1-7*, an ETR1 null mutant, that contained either a genomic transgene of *ETR1* (*gETR1*) or a cDNA of *ETR1* (*cETR1*) under the control of native ETR1 promoter (2.2 kb). We also generated *etr1-7* lines that were transformed with the cDNA of *ETR1*^{H353Q} (*cETR1*^{H353Q}), in which Gln (Q) replaces the only phosphorylatable His (H) 353 (Moussatche and Klee, 2004). For our analysis, each of several transgenic lines was selected both for a homozygous single transgene insertion and for similar transgenic protein expression at T3 generation (Fig. 1B; see Supplemental Fig. S1).

First, we reassessed whether ETR1 HK activity is involved in ethylene signaling by observing etiolated seedlings growing on the Murashige and Skoog media containing 1% (w/v) Suc and 10 μM of 1-aminocyclopropane-1-carboxylic acid (ACC), which is the immediate precursor of ethylene. Wild-type, *etr1-7*, and transgenic *gETR1*, *cETR1*^{H353Q}, and *cETR1* seedlings displayed a typical triple response: inhibition of hypocotyl and root growth, exaggeration of apical hook formation, and hypocotyl thickening (Fig. 1, C and D). As expected, the *etr1-1* and *etr1-2* alleles showed strong and weak insensitivity to ACC, respectively (Bleecker et al., 1988; Hall et al., 1999).

An indifferent response among the transgenic lines at a saturating dose of ACC (10 μM) could be caused by the functional redundancy of receptors in ethylene signaling (Hua and Meyerowitz, 1998). To uncover ETR1 function among other redundant members, we observed etiolated seedlings growing on 1 μM of ACC (Alonso et al., 2003). Again, hypocotyls and roots of wild-type, *etr1-7*, and transgenic seedlings displayed similar growth inhibition (Fig. 1, C and D). Thus, our results with quantitative triple response assays support the notion that ETR1 HK activity is dispensable in ethylene signaling (Gamble et al., 2002; Wang et al., 2003; Binder et al., 2004; Qu and Schaller, 2004).

Next, we evaluated the role of ETR1 HK activity in response to ambient and/or endogenous ethylene. Wild-type, transgenic *gETR1*, and *cETR1* etiolated seedlings growing without ACC addition displayed normal hypocotyl elongation (Fig. 1, C and D). However, dark-grown seedlings appear to be sensitive to the ambient/endogenous ethylene, because seedlings exhibited enhanced hypocotyl elongation in the presence of silver. Silver has been shown to competitively inhibit ethylene receptor function (Rodríguez et al., 1999).

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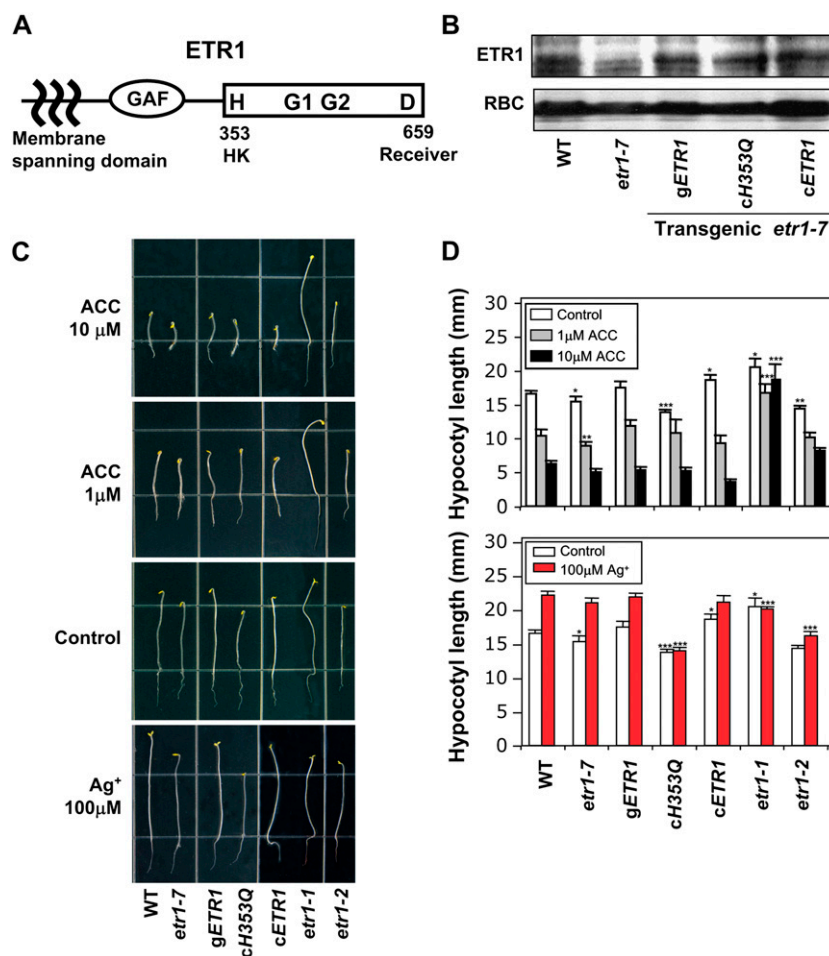


Figure 1. Structure and functions of ETR1 HK activity for plant growth promotion in the dark. A, The diagram depicts putative functional domains of ETR1. B, Expression of ETR1 proteins was detected by protein-blot analysis using anti-ETR1 antibody (Santa Cruz Biotech). Transgenic lines are *etr1-7* plants transformed with the wild-type *gETR1*, the cDNA *ETR1* gene containing a H353Q mutation (*cH353Q*), and the wild-type *cETR1*. For comparison, proteins of wild type (ecotype Columbia) and *etr1-7* (*etr1* null) were analyzed together. To detect the membrane protein ETR1, plants (14 d) were ground in 50 mM HEPES-KOH buffer, pH 7.6, containing 2 mM dithiothreitol, 2 mM EDTA, 10 mM β -glycerophosphate, 20% (v/v) glycerol, 1 \times complete protease inhibitor mix (Roche), and 1% (v/v) Triton X-100, and incubated on ice for 30 min. After removing tissue debris, protein loading buffer was added. The extracts were incubated at 65°C for 15 min and then 37°C for 30 min before running on 10% (w/v) SDS-polyacrylamide gels. The Rubisco (RBC) was shown as a protein loading control (Yanagisawa et al., 2003). C, Phenotypes of 4-d-old seedling growth in the dark. Seedlings were grown under saturating dosage of ACC (10 μ M), subsaturating dosage of ACC (1 μ M), ambient/endogenous ethylene (control), and ethylene-free (100 μ M Ag^+) conditions. For comparison, wild-type, *etr1-7* (*etr1* null), *etr1-1* (strong ethylene-insensitive), and *etr1-2* (weak ethylene-insensitive) seedlings were grown together with the transgenic *etr1-7* lines. D, Quantitative analysis of hypocotyl length of 4-d-old etiolated seedlings. Values are means with SD, $n = 30$. Asterisks over bars indicate differences between wild type and mutant with statistical significance at * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ (t test). [See online article for color version of this figure.]

Indeed, *etr1-1* seedlings, in which ethylene binding is abolished, displayed enhanced hypocotyl elongation without silver but did not show further elongation with silver, demonstrating its strong ethylene insensitivity. The *etr1-2* displayed slow hypocotyl elongation without silver and enhanced hypocotyl elongation with silver, which is consistent with its weak ethylene insensitivity.

We note that *etr1-7*, in comparison to wild type, displayed relatively slow hypocotyl elongation in the absence of silver with statistical confidence (* $P < 0.05$) and fully enhanced hypocotyl elongation in the

presence of silver (Fig. 1, C and D). This observation may implicate a role for ETR1 in plant growth that is independent of ethylene signaling (Hua and Meyerowitz, 1998), or the seedlings without ETR1 may be hypersensitive to ethylene (Cancel and Larsen, 2002).

Interestingly, transgenic *cETR1*^{H353Q} seedlings exhibited even slower hypocotyl elongation than *etr1-7* without silver and lacked further hypocotyl elongation with silver. This indicates that the growth defect caused by the loss of ETR1 HK activity is not because of the increased ethylene production or ethylene responsiveness.

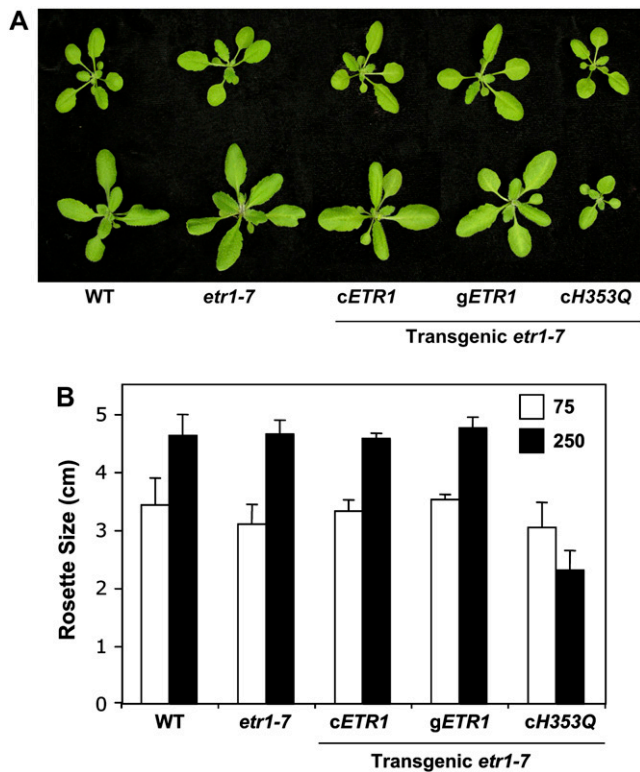


Figure 2. Functions of ETR1 HK activity for plant growth promotion under light. A, Phenotypes of the wild-type, *etr1-7*, and transgenic *etr1-7* plants grown in the cycle of 16-h light and 8-h dark with light intensity of 75 $\mu\text{E m}^{-2} \text{s}^{-1}$ (top) or 250 $\mu\text{E m}^{-2} \text{s}^{-1}$ (bottom) for 21 d. B, Quantitative analysis (rosette diameter) of plant growth phenotypes under normal (75) and high (250) light intensity conditions. Values are means with SD, $n = 20$.

Because plant growth in either the light or darkness is mechanically distinct, we decided to also observe growth of transgenic plants under light. With the exception of *cETR1^{H353Q}*, all other lines were indistinguishable from wild type under normal growth conditions (the cycle of 16-h light and 8-h dark with the light intensity of 75 $\mu\text{E m}^{-2} \text{s}^{-1}$). The *cETR1^{H353Q}* lines showed a minor reduction in rosette size (Fig. 2, A [top] and B). In growth-promoting light conditions (the light intensity of 250 $\mu\text{E m}^{-2} \text{s}^{-1}$), wild-type, *etr1-7*, and transgenic *cETR1* and *gETR1* plants exhibited relatively bigger rosette size (Fig. 2, A [bottom] and B). However, transgenic *cETR1^{H353Q}* plants failed to promote such enhanced rosette growth, indicating that ETR1 HK activity is also involved in growth promotion under light. It will be interesting to further test if the light signaling-implicated GAF domain of ETR1 plays an additional role in the high light-driven growth promotion.

Because ethylene receptors have at least partial functional redundancy, the growth defects of *cETR1^{H353Q}* suggest that ETR1^{H353Q} has a dominant negative effect on the other ethylene receptors. This may indicate the existence of heteromeric receptor complexes. Currently,

a receptor complex containing an ETR1 homodimer and CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) has been biochemically characterized; however, other partners remain to be identified (Schaller et al., 1995; Chen et al., 2002; Gao et al., 2003). Recently, a genetic screen for suppressors of *etr1-2* identified the recessive mutant *reversion to ethylene sensitivity 1*, which acts upstream of ETR1 (Resnick et al., 2006) and is a potential component of the ETR1 receptor complex.

To gain insight into the molecular mechanism(s) underlying the growth defects associated with the loss of ETR1 HK activity, we monitored gene expression in etiolated seedlings using quantitative reverse transcription-PCR. Typically, a receptor HK is a signal input module of a two-component system (Hwang et al., 2002). Thus, we examined marker gene expression that is induced by the two-component system as well as by ethylene signaling. The gene expression of *ERF1* is a diagnostic feature of ethylene signaling (Solano et al., 1998) and was significantly higher in *etr1-7* but abolished in the dominant ethylene-insensitive *etr1-1* compared with wild type (Fig. 3A). The *ERF1* gene expression was similarly suppressed in both transgenic *cETR1* and *cETR1^{H353Q}*, supporting the contention that the growth defect in the transgenic *cETR1^{H353Q}* is not caused by ethylene responsiveness but by the lack of growth promotion resulting from the loss of HK activity.

The gene expression of the A-type *ARR5* is a hallmark of two-component system activity (D'Agostino et al., 2000) and was relatively higher in transgenic *cETR1* in comparison to *etr1-7*, *cETR1^{H353Q}*, and wild type (Fig. 3A). The *ARR5* gene expression was significantly higher in *etr1-1*. Mature plant growth of *etr1-1* is also greatly enhanced with a higher level of the ETR1 mutant protein (*etr1-1*: ETR1C65Y) accumulation, which is incompetent in ethylene signaling (Zhao et al., 2002). Most likely, the induced *ARR5* gene expression in *etr1-1* resulted from the two-component system activated by the *etr1-1*.

We also detected a high level of the B-type *ARR1* expression in *etr1-1* (see Supplemental Fig. S2). Currently, the function of increased *ARR1* gene expression is unknown; however, it may balance the constitutive A-type ARR activity, which plays a repressor role in the two-component system (Hwang et al., 2002).

To substantiate the downstream molecular mechanism of ETR1 HK activity, we examined whether the kinase activity could modulate two-component system by taking advantage of the well-established *ARR6* promoter fused with luciferase reporter (*ARR6-LUC*; Hwang and Sheen, 2001). We generated constructs of an ETR1 wild type (*ETR1WT*), inactive forms (*H353Q* and *D659A*), and a constitutive active form (*D659E*). Individual construct was cotransfected with *ARR6-LUC* to the mesophyll protoplasts generated from *etr1-7* leaf tissues. A minimum level of the B-type ARR positive mediator (*ARR10*) was also cotransfected to sensitize the ethylene-sensitive *etr1-7* cells to the two-component system activity. Both *ETR1WT* and *D659E* induced

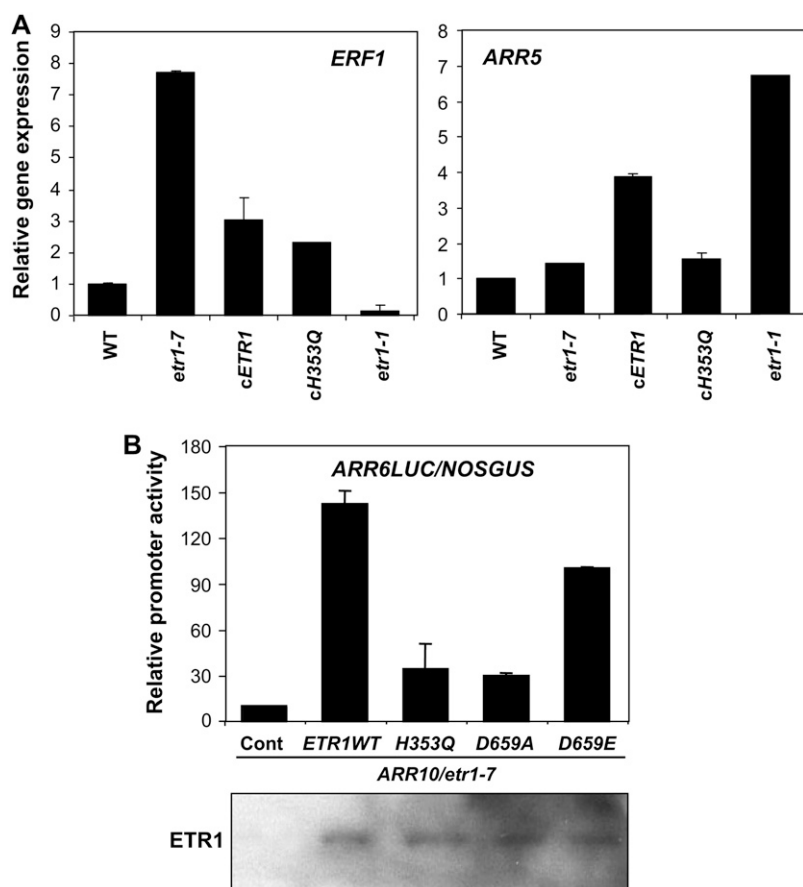


Figure 3. Functions of ETR1 HK activity in the activation of two-component system. A, Relative expressions of marker genes (*ERF1*, At3G23240; *ARR5*, At3g48100) in the 4-d-old etiolated seedlings growing at the ambient/endogenous ethylene condition. Values are means with SD ($n = 3$) obtained by using quantitative reverse transcription-PCR and calculated relatively to those of wild type. Expression level of *eIF4a* (At3g13920) gene was served as a control. B, The ARR6-LUC reporter activity induced by various ETR1 effectors. Values are means with SD ($n = 5$). Effector constructs including ETR1WT, inactive form *ETR1* genes containing H353Q mutation and D659A mutation, and an activated form *ETR1* gene containing D659E mutation were generated by PCR and site-specific mutagenesis in plant expression vectors. The designated constructs were cotransfected to leaf mesophyll protoplasts generated from *etr1-7* leaf tissues, and the LUC reporter assay was performed as before (Hwang and Sheen, 2001; Sheen Web site, <http://genetics.mgh.harvard.edu/sheenweb>). The effector (*ETR1* variants), mediator (*ARR10*, At4g31920), reporter (*ARR6LUC*), and internal control reporter (*NOSGUS*) constructs were transfected in the ratio of 4:1:4:1. Expressions of ETR1 proteins were shown by protein-blot analysis using anti-ETR1 antibody.

the ARR6-LUC reporter activity in a similar fashion, whereas such reporter induction was greatly lowered with H353Q and D659A (Fig. 3B). In summary, ETR1 appears to activate two-component system through the conserved HK (H353) and response regulator (D659) residues.

Because ETR1WT was as potent as D659E for reporter induction, the ETR1WT appeared to be activated upon its expression. This is consistent with the previous observation that ETR1 is autophosphorylated without its ligand binding *in vitro* (Gamble et al., 1998, 2002; Moussatche and Klee, 2004). Therefore, the activation mechanism of ETR1 HK appears distinct from other Arabidopsis HKs functioning in cytokinin signaling, of which activities are largely dependent on the ligand binding (Hwang and Sheen, 2001).

Here we have shown that ETR1 HK activity triggers the two-component system and promotes plant growth in Arabidopsis. It remains to be determined if the two-component system activated by ETR1 HK activity contributes to the plant growth promotion. It would also be interesting to know if ETR1 HK activity switches on and off in the absence and presence of ethylene, respectively, to coordinate plant growth promotion and inhibition. Further elucidation of molecular mechanisms underlying ETR1 action will improve our understanding of ethylene functions in plant growth and developments.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Consistent phenotypes of independent transgenic lines.

Supplemental Figure S2. Relative expressions of *ARR1*.

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