Arabidopsis RTE1 Is Essential to Ethylene Receptor ETR1 Amino-Terminal Signaling Independent of CTR1[1][C]

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The Arabidopsis (*Arabidopsis thaliana*) ethylene receptor Ethylene Response1 (ETR1) can mediate the receptor signal output via its carboxyl terminus interacting with the amino (N) terminus of Constitutive Triple Response1 (CTR1) or via its N terminus (etr1$^{1\text{-}349}$ or the dominant ethylene-insensitive etr1-1$^{1\text{-}349}$) by an unknown mechanism. Given that CTR1 is essential to ethylene receptor signaling and that overexpression of *Reversion To Ethylene Sensitivity1* (*RTE1*) promotes ETR1 N-terminal signaling, we evaluated the roles of CTR1 and RTE1 in ETR1 N-terminal signaling. The mutant phenotype of *ctr1-1* and *ctr1-2* was suppressed in part by the transgenes *etr1*$^{1\text{-}349}$ and *etr1-1*$^{1\text{-}349}$, with *etr1-1*$^{1\text{-}349}$ conferring ethylene insensitivity. Coexpression of *35S:RTE1* and *etr1*$^{1\text{-}349}$ conferred ethylene insensitivity in *ctr1-1*, whereas suppression of the *ctr1-1* phenotype by *etr1*$^{1\text{-}349}$ was prevented by *rte1-2*. Thus, RTE1 was essential to ETR1 N-terminal signaling independent of the CTR1 pathway. An excess amount of the CTR1 N terminus CTR1$^{7\text{-}560}$ prevented ethylene receptor signaling, and the *CTR1*$^{7\text{-}560}$ overexpressor *CTR1-Nox* showed a constitutive ethylene response phenotype. Expression of the ETR1 N terminus suppressed the *CTR1-Nox* phenotype. *etr1*$^{1\text{-}349}$ restored the ethylene insensitivity conferred by dominant receptor mutant alleles in the *ctr1-1* background. Therefore, ETR1 N-terminal signaling was not mediated by full-length ethylene receptors; rather, full-length ethylene receptors acted cooperatively with the ETR1 N terminus to mediate the receptor signal independent of CTR1. ETR1 N-terminal signaling may involve RTE1, receptor cooperation, and negative regulation by the ETR1 carboxyl terminus.

The gaseous plant hormone ethylene is perceived by a small family of ethylene receptors. Arabidopsis (*Arabidopsis thaliana*) has five ethylene receptors that are structurally similar to prokaryotic two-component histidine kinase (HK) proteins. Mutants defective in multiple ethylene receptor genes show a constitutive ethylene response phenotype, which indicates a negative regulation of ethylene responses by the receptor genes (Hua and Meyerowitz, 1998).

The receptor N terminus has three or four transmembrane domains that bind ethylene. The GAF (for cGMP-specific phosphodiesterases, adenylyl cyclases, and FhlA) domain, which follows the transmembrane helices, mediates noncovalent receptor heterodimerization and may have a role in receptor cooperation (Gamble et al., 2002; O'Malley et al., 2005; Xie et al., 2006; Gao et al., 2008). The subfamily I receptors Ethylene Response1 (ETR1) and Ethylene Response Sensor1 (ERS1) have a conserved HK domain following the GAF domain. For subfamily II members ETR2, Ethylene Insensitive4 (EIN4), and ERS2, the HK domain is less conserved, and they lack most signature motifs essential for HK activity (Chang et al., 1993; Gamble et al., 1998; Hua et al., 1998; Qu and Schaller, 2004; Xie et al., 2006). Among the five receptors, ETR1, ETR2, and EIN4 have a receiver domain following the HK domain. The ETR1 HK domain may have a role in mediating the receptor signal to downstream components, and the HK activity facilitates the ethylene signaling (Clark et al., 1998; Huang et al., 2003; Hall et al., 2012). The receiver domain can dimerize and could involve receptor cooperation (Müller-Dieckmann et al., 1999). However, differential receptor cooperation occurs between the receiver domain-lacking ERS1 and the other ethylene receptors, which does not support the hypothesis that the domains involve receptor cooperation (Liu and Wen, 2012).

Acting downstream of the ethylene receptors is Constitutive Triple Response1 (CTR1), a MEK kinase (mitogen-activated protein kinase kinase kinase) with Ser/Thr kinase activity, and the kinase domain locates at the C terminus. The CTR1 N terminus does not share sequence similarity to known domains and can physically interact with the ethylene-receptor HK domain (Clark et al., 1998; Huang et al., 2003). *ctr1* mutants showing attenuated CTR1 kinase activity or the ETR1-CTR1 association exhibit various degrees of the

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constitutive ethylene-response phenotype. For example, the *ctr1-1* and *ctr1^{btk}* mutations result from the D694E and E626K substitutions, respectively, in the CTR1 kinase domain, and *ctr1-1* shows a stronger ethylene-response phenotype than *ctr1^{btk}*, with *ctr1-1* having much weaker kinase activity than *ctr1^{btk}* (Kieber et al., 1993; Huang et al., 2003; Ikeda et al., 2009). The *ctr1-8* mutation results in the G354E substitution that prevents the ETR1-CTR1 association, and the mutant exhibits a constitutive ethylene-response phenotype. Overexpression of the CTR1 N terminus CTR1$^{7-560}$, which is responsible for interaction with ethylene receptors, leads to constitutive ethylene responses, possibly by titrating out available ethylene receptors (Kieber et al., 1993; Huang et al., 2003). These studies suggest that CTR1 kinase activity and the interaction of CTR1 with the receptor HK domain may be important to the ethylene receptor signal output in suppressing constitutive ethylene responses.

Although the ETR1-CTR1 interaction via the HK domain is essential to the ethylene receptor signal output, evidence suggests that the ETR1 receptor signal output can also be independent of the HK activity or domain. The *etr1 ers1* loss-of-function mutant displays extreme growth defects. The *etr1*[*HGG*] mutation inactivates ETR1 HK activity, and expression of the *getr1*[*HGG*] transgene rescues the *etr1 ers1* growth defects, which indicates a lack of association of ETR1 receptor signaling and its kinase activity (Wang et al., 2003). The dominant *etr1-1* mutation results in the C65Y substitution and confers ethylene insensitivity (Chang et al., 1993), and the expression of the HK domain-lacking etr1$^{1-349}$ and ethylene-insensitive etr1-1$^{1-349}$ isoforms partially suppresses the growth defects of *etr1 ers1-2*. Loss-of-function mutations of subfamily II members do not affect *etr1-1$^{1-349}$* functions. Therefore, etr1-1$^{1-349}$ predominantly cooperates with subfamily I receptors to mediate the ethylene receptor signal output (Xie et al., 2006). Biochemical and transformation studies showing that ethylene receptors can form heterodimers and that each receptor is a component of high-molecular-mass complexes explain how ethylene receptors may act cooperatively (Gao et al., 2008; Gao and Schaller, 2009; Chen et al., 2010).

Reversion To Ethylene Sensitivity1 (RTE1), a Golgi/endoplasmic reticulum protein, was isolated from a suppressor screen of the dominant ethylene-insensitive *etr1-2* mutation. The cross-species complementation of the *rte1-2* loss-of-function mutation by the rice (*Oryza sativa*) *RTE Homolog1* (*OsRTH1*) suggests a conserved mechanism that modulates the ethylene receptor signaling across higher plant species (Zhang et al., 2012). *RTE1* and *OsRTH1* overexpression led to ethylene insensitivity in wild-type Arabidopsis but not the *etr1-7* loss-of-function mutant, and expression of *etr1$^{1-349}$* restored ethylene insensitivity with *RTE1* overexpression in *etr1-7* (Resnick et al., 2006; Zhou et al., 2007; Zhang et al., 2010). Coimmunoprecipitation of epitope-tagged ETR1 and RTE1 and Trp fluorescence spectroscopy revealed the physical interaction of RTE1 and ETR1 (Zhou et al., 2007; Dong et al., 2008, 2010). Therefore, RTE1 may directly promote ETR1 receptor signal output through the ETR1 N terminus, but whether RTE1 has an essential role in ETR1 N-terminal signaling remains to be addressed.

Currently, the biochemical nature of the ethylene receptor signal is unknown, and the underlying mechanisms of mediation of the ethylene receptor signal output remain uninvestigated. Genetic and biochemical studies suggest that activation of CTR1 by ethylene receptors may suppress constitutive ethylene responses; upon ethylene binding, the receptors are converted to an inactive state and fail to activate CTR1, and the suppression of ethylene responses by CTR1 is alleviated (Hua and Meyerowitz, 1998; Klee, 2004; Wang et al., 2006; Hall et al., 2007). However, this model does not address how the ETR1 N terminus, which does not have the CTR1-interacting site, mediates the receptor signal to suppress constitutive ethylene responses. The receptor signal of the truncated etr1 isoforms may be mediated by other full-length ethylene receptors and then activate CTR1; alternatively, the ETR1 N-terminal signal may be mediated by a pathway independent of CTR1 (Gamble et al., 2002; Qu and Schaller, 2004; Xie et al., 2006). Results showing that mutants defective in multiple ethylene receptor genes exhibit a more severe ethylene-response phenotype than *ctr1* and that *ctr1* mutants are responsive to ethylene support the presence of a CTR1-independent pathway (Hua and Meyerowitz, 1998; Cancel and Larsen, 2002; Huang et al., 2003; Liu et al., 2010).

In this study, we investigated whether mediation of ETR1 N-terminal signaling is independent of CTR1 and whether RTE1 is essential to the CTR1-independent ETR1 N-terminal signaling. The ETR1 N-terminal signaling was not mediated via other full-length ethylene receptors, but the signal of full-length ethylene receptors could be mediated by the ETR1 N terminus independent of CTR1. The ETR1 C terminus may inhibit ETR1 N-terminal signaling, whereby deletion of the C terminus facilitates N-terminal signaling. We propose a model for the possible modulation of ETR1 receptor signaling.

RESULTS

Expression of *etr1$^{1-349}$* or *etr1-1$^{1-349}$* Partly Suppresses the Constitutive Ethylene Response Phenotype of *ctr1-1*

To investigate whether the ETR1 N terminus can mediate the ethylene receptor signal to constitutively suppress ethylene responses in the absence of CTR1, we expressed transgenes encoding etr1$^{1-349}$ and the ethylene-insensitive etr1-1$^{1-349}$ in the *ctr1-1* loss-of function mutant. If ETR1 N-terminal signaling was mediated independent of CTR1, expression of the transgenes might suppress the constitutive ethylene response in *ctr1-1*. The dominant nature of etr1-1$^{1-349}$ can facilitate

the study of ETR1 N-terminal signaling in *ctr1-1*. Given that the *etr1-1* mutation confers a gain of function, we examined the effects of $etr1^{1\text{-}349}$ expression on the *ctr1-1* mutant phenotype to address ETR1 N-terminal signaling in the absence of CTR1.

The structures and domains of the etr1 and ctr1 isoforms are shown in Figure 1A. *ctr1-1* lines that expressed the *ETR1p:etr1-1*$^{1\text{-}349}$ or *ETR1p:etr1*$^{1\text{-}349}$ transgene (designated *etr1-1*$^{1\text{-}349}$ and *etr1*$^{1\text{-}349}$, respectively) were obtained by transformation or genetic

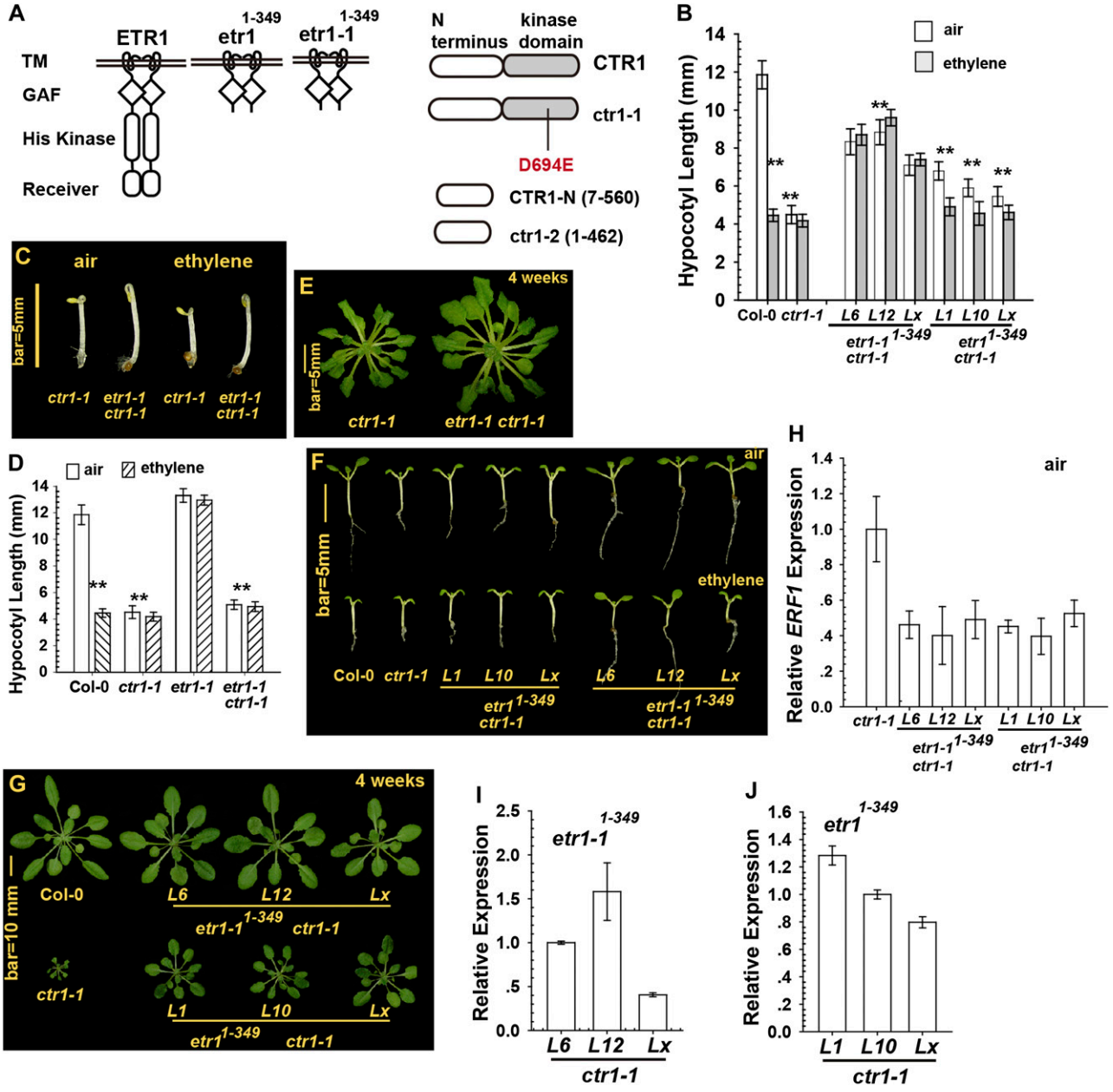

Figure 1. Expression of the ETR1 N terminus partially suppresses constitutive ethylene responses mediated by *ctr1-1*. A, Structure and mutations of ETR1 and CTR1 isoforms described in this study. TM, Transmembrane domain. B, Seedling hypocotyl length of *ctr1-1* and *ctr1-1* expressing *etr1-1*$^{1\text{-}349}$ or *etr1*$^{1\text{-}349}$. C and D, Phenotype (C) and hypocotyl length (D) of etiolated seedlings of *ctr1-1* and *etr1-1 ctr1-1*. E, Rosette phenotype of *ctr1-1* and *etr1-1 ctr1-1*. F and G, Phenotype of light-grown seedlings (F) and rosettes (G) of *ctr1-1* and *ctr1-1* expressing *etr1*$^{1\text{-}349}$ or *etr1-1*$^{1\text{-}349}$. H, *ERF1* expression in *ctr1-1* and *ctr1-1* expressing the ETR1 N terminus. I and J, Measurement of the relative transgene expression for *etr1-1*$^{1\text{-}349}$ (I) and *etr1*$^{1\text{-}349}$ (J) of corresponding *ctr1-1* transformation lines. *etr1*$^{1\text{-}349}$ and *etr1-1*$^{1\text{-}349}$ denote the *etr1*$^{1\text{-}349}$ and *etr1-1*$^{1\text{-}349}$ transgenes, respectively, that are driven by the native *ETR1* promoter. *L* indicates the transformation lines used in this assay. Data are means ± SD ($n \geq 30$). ** $P < 0.01$ by Student's *t* test. [See online article for color version of this figure.]

crossing. Here, *Lx* refers to the line that was obtained from the genetic cross of *ctr1-1* and the previously obtained *etr1-7 ers1-2* lines that carry *etr1-1*$^{1-349}$ or *etr1*$^{1-349}$ (Xie et al., 2006).

As expected, dark-grown *ctr1-1* seedlings showed a constitutive ethylene-response phenotype, with a short hypocotyl and root and an exaggerated apical hook in the absence of ethylene treatment. *ctr1-1* was marginally responsive to ethylene, and ethylene-treated *ctr1-1* seedlings had a shorter hypocotyl than air-grown seedlings (Fig. 1, B and C; Student's *t* test, $P < 0.01$). Interestingly, the expression of the ETR1 N terminus rescued in part the *ctr1-1* seedling growth defects. Air-grown, etiolated *etr1-1*$^{1-349}$ *ctr1-1* and *etr1*$^{1-349}$ *ctr1-1* seedlings had a longer seedling hypocotyl than *ctr1-1* seedlings. Ethylene treatment inhibited the etiolated-seedling hypocotyl growth of *etr1*$^{1-349}$ *ctr1-1* but not *etr1-1*$^{1-349}$ *ctr1-1* (Fig. 1B; Fisher's LSD, $\alpha = 0.01$).

We examined whether the dominant ethylene-insensitive *etr1-1* allele has the same effect as *etr1-1*$^{1-349}$ on suppression of the *ctr1-1* mutant phenotype. The *etr1-1 ctr1-1* seedlings phenotypically resembled but were slightly longer than *ctr1-1* seedlings regardless of ethylene treatment (Student's *t* test, $P < 10^{-6}$; Fig. 1, C and D). Consistently, at the adult stage, *ctr1-1* growth defects were slightly suppressed by *etr1-1* (Fig. 1E).

Grown under light, *etr1-1*$^{1-349}$ had a greater effect than *etr1*$^{1-349}$ on suppressing the *ctr1-1* seedling growth defects. Ethylene treatment inhibited the seedling growth of the wild type (ecotype Columbia [Col-0]), *ctr1-1*, and *ctr1-1* lines expressing the *etr1*$^{1-349}$ transgene. As expected, *etr1-1*$^{1-349}$ *ctr1-1* lines showed minor growth inhibition with ethylene treatment because *etr1-1*$^{1-349}$ expression conferred ethylene insensitivity (Fig. 1F). At the adult stage, *ctr1-1* growth defects were substantially suppressed by *etr1-1*$^{1-349}$ and *etr1*$^{1-349}$ (Fig. 1G). These results suggested that the receptor signal output was mediated by etr1$^{1-349}$ in the absence of CTR1 in suppressing constitutive ethylene responses and was prevented by ethylene treatment. The etr1-1$^{1-349}$ signaling was independent of CTR1 and was not affected by ethylene because ethylene binding was prevented by the C65Y mutation (Wang et al., 2006).

Ethylene Response Factor1 (*ERF1*) is a primary target of the ethylene signal with expression induced by ethylene (Solano et al., 1998). *ERF1* is thus ideal for measuring the degree of ethylene responsiveness. Quantitative reverse transcription (qRT)-PCR revealed *ERF1* expression attenuated by about 60% in *ctr1-1* lines that expressed *etr1-1*$^{1-349}$ or *etr1*$^{1-349}$ (Fig. 1H). These results suggest an association of growth recovery and suppression of the constitutive ethylene response in air-grown *ctr1-1* plants by the transgenes.

The *etr1*$^{1-349}$ and *etr1-1*$^{1-349}$ transgenes were each introduced into *ctr1-1* by genetic crossing or transformation, and their expression levels were measured to determine their effect on the suppression of the *ctr1-1* mutant phenotype. qRT-PCR revealed the *etr1-1*$^{1-349}$ transgene to be expressed at a lower level in the *ctr1-1* transformation line *Lx* than in the other lines (Fig. 1I). The expression of *etr1*$^{1-349}$ in the three transformation lines varied, but the difference was minor (±20% relative to that of line *L10*; Fig. 1J), which may explain why the *etr1-1*$^{1-349}$ *ctr1-1* line *Lx* exhibited stronger growth inhibition than the other *etr1-1*$^{1-349}$ *ctr1-1* lines at the seedling stage.

Because *ctr1-1* did not completely suppress the effect of the *etr1-1* mutation and *ctr1-1* was slightly ethylene responsive, *ctr1-1* might not be a null mutation; alternatively, the receptor signal might be mediated in part via an alternative pathway independent of CTR1. Our data show that the growth defects and the constitutive ethylene response caused by the *ctr1-1* mutation were suppressed in part by expression of the ETR1 N terminus and that expression of *etr1-1*$^{1-349}$ but not *etr1*$^{1-349}$ conferred ethylene insensitivity in *ctr1-1*. These results support a model in which ETR1 N-terminal signaling is mediated independently of CTR1.

The Constitutive Ethylene Response of *ctr1-2* Is Partly Suppressed by Transgenes Encoding the ETR1 N Terminus

The *ctr1-1* mutation substantially weakens the Ser/Thr kinase activity and does not prevent ctr1-1 protein expression (Huang et al., 2003). *ctr1-1* may not be a null mutation, so it may mediate a small amount of ethylene receptor signal output. The *ctr1-2* mutation results in a 17-bp deletion in the *CTR1* coding region, and *ctr1-2* may encode a truncated protein of 462 residues that lacks the kinase domain (Huang et al., 2003; Fig. 1A). We investigated whether the constitutive ethylene response caused by the *ctr1-2* mutation can be suppressed by expression of the ETR1 N terminus to determine whether ETR1 N-terminal signaling requires CTR1.

We introduced *etr1-1*$^{1-349}$ and *etr1*$^{1-349}$ transgenes into *ctr1-2* by genetic crossing from line *Lx* so that the corresponding transgenes were expressed at the same locus in *ctr1-1* and *ctr1-2*. Etiolated *ctr1-2* seedlings had a short hypocotyl when grown in air and with ethylene treatment; notably, ethylene treatment slightly inhibited *ctr1-2* seedling growth (Student's *t* test, $P < 0.01$). Expression of *etr1*$^{1-349}$ or *etr1-1*$^{1-349}$ in part suppressed the *ctr1-2* growth defects in air, and *ctr1-2* seedlings with each transgene had longer hypocotyls than *ctr1-2* seedlings alone (Fisher's LSD, $\alpha = 0.01$). As expected, ethylene treatment inhibited the seedling growth of *etr1*$^{1-349}$ *ctr1-2* but not *etr1-1*$^{1-349}$ *ctr1-2* (Fig. 2, A and B). Consistently, both etr1 isoforms suppressed the growth inhibition of light-grown *ctr1-2* seedlings. *etr1*$^{1-349}$ *ctr1-2* and *etr1-1*$^{1-349}$ *ctr1-2* seedlings showed larger cotyledons and longer hypocotyls and roots than *ctr1-2* when grown in air. As expected, ethylene treatment inhibited the seedling growth of *ctr1-2* and *etr1*$^{1-349}$ *ctr1-2* but not *etr1-1*$^{1-349}$ *ctr1-2* (Fig. 2C). At the adult stage, *ctr1-2* showed strong growth inhibition and the rosette was extremely small; rosettes were larger for both *etr1*$^{1-349}$ *ctr1-2* and *etr1-1*$^{1-349}$ *ctr1-2* than for *ctr1-2* (Fig. 2D). *ERF1* expression was attenuated by about 40% and 50% in *ctr1-2* plants with *etr1*$^{1-349}$ and *etr1-1*$^{1-349}$, respectively (Fig. 2E).

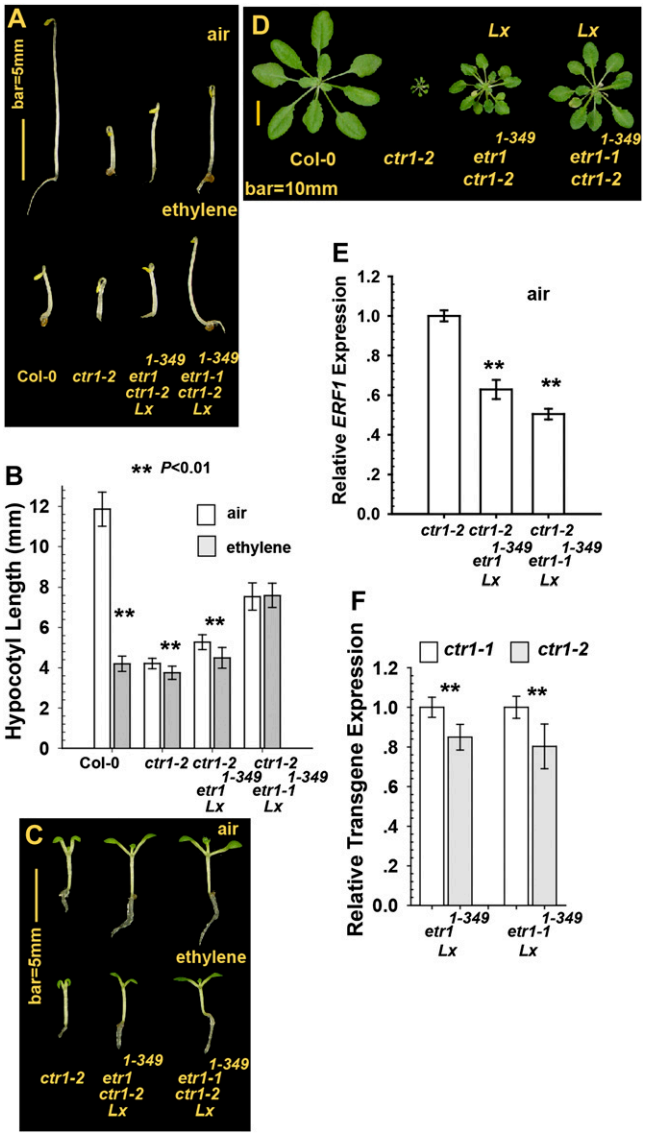

Figure 2. Expression of the ETR1 N terminus partially rescues the growth defects of *ctr1-2*. A and B, Phenotype (A) and hypocotyl measurement (B) of etiolated seedlings of *ctr1-2* and *ctr1-2* expressing $etr1^{1-349}$ or $etr1\text{-}1^{1-349}$. C and D, Phenotype of light-grown seedlings (C) and rosettes (D) of *ctr1-2* and *ctr1-2* expressing $etr1^{1-349}$ or $etr1\text{-}1^{1-349}$. E, Relative *ERF1* expression level of *ctr1-2* and *ctr1-2* expressing the truncated etr1 isoforms. F, Relative transgene expression in the transgene donor (*ctr1-1*, *Lx*) and recipient (*ctr1-2*, *Lx*). *Lx* indicates the transformation lines. Data are means ± SD. ** $P < 0.01$ by Student's *t* test (B and F) and Fisher's LSD (E). [See online article for color version of this figure.]

The transgenes were introduced by genetic crossing into *ctr1-2*. qRT-PCR to determine whether the expression of the transgenes would vary in transgene donors (*ctr1-1* lines) and recipients (*ctr1-2* lines) revealed the expression of the transgenes to be slightly lower in *ctr1-2* than in *ctr1-1* (Fig. 2F; Student's *t* test, $P < 0.01$). However, the reduction was minor (about 20%) and did not contradict our conclusion that expression of the truncated etr1 isoforms in part suppressed *ctr1-2* growth inhibition and attenuated the constitutive ethylene response.

Our results were in agreement with the mediation of the ETR1 N-terminal signal output to suppress the constitutive ethylene response being independent of the wild-type CTR1 and ethylene binding to the ETR1 N terminus preventing the N-terminal receptor signal output. In addition, similar to *ctr1-1*, *ctr1-2* was slightly ethylene responsive, which implied an alternative signal transduction pathway.

The Suppression of the *ctr1* Phenotype by the ETR1 N Terminus Occurs Independently of Wild-Type *ETR1*

We showed that expression of $etr1^{1-349}$ or $etr1\text{-}1^{1-349}$ suppressed the constitutive ethylene-response phenotype of *ctr1-1* and *ctr1-2*, both with the wild-type *ETR1*. Given that the full-length ETR1 and the truncated isoforms physically interact and may cooperate (Xie et al., 2006; Gao et al., 2008), we evaluated whether suppression of the *ctr1* mutant phenotype resulted from coexpression of the wild-type and truncated ETR1 isoforms.

The *etr1-7* mutation results in the W74stop substitution and is believed to be amorphic (Hua and Meyerowitz, 1998; Xie et al., 2006). The $etr1\text{-}1^{1-349}$ and $etr1^{1-349}$ transgenes were introduced into *etr1-7 ctr1-1* and *etr1-7 ctr1-2*, respectively, by genetic crossing from the transgene donors as shown in Figures 1 and 2, so that each transgene was expressed at the same locus in the corresponding transgene donor and recipient. Transformation lines labeled with the same number carry the transgene from a common transgene donor. qRT-PCR revealed similar expression of the transgene from the same donor in different genotypes. One exception was that $etr1\text{-}1^{1-349}$ expression was slightly reduced (by 20%) in *etr1-7 ctr1-1* $etr1\text{-}1^{1-349}$ line *L6* (Fig. 3, A and B).

The hypocotyl was shorter for ethylene-treated, etiolated *etr1-7 ctr1-1* than air-grown seedlings (Fig. 3, C and D). The constitutive ethylene-response phenotype was stronger for dark-grown *etr1-7 ctr1-1* than *ctr1-1* seedlings, regardless of ethylene treatment (Figs. 1B and 3D). On the basis of the seedling triple-response phenotype and hypocotyl length, our data show that expression of $etr1\text{-}1^{1-349}$ or $etr1^{1-349}$ suppressed the growth inhibition of *etr1-7 ctr1-1* and *etr1-7 ctr1-2* to a large extent. As expected, ethylene treatment inhibited the hypocotyl elongation of *etr1-7 ctr1-1* and *etr1-7 ctr1-2* seedlings that expressed $etr1^{1-349}$ (Student's *t* test, $P < 0.01$) but not those that expressed $etr1\text{-}1^{1-349}$ (Fig. 3, C and D).

Grown under light, seedlings of *ctr1-1*, *ctr1-2*, *etr1-7 ctr1-1*, and *etr1-7 ctr1-2* showed severe growth inhibition, small and compact cotyledons, and short hypocotyls and primary roots. As expected, expression of $etr1\text{-}1^{1-349}$ or $etr1^{1-349}$ substantially suppressed the mutant growth defects, and the transformation

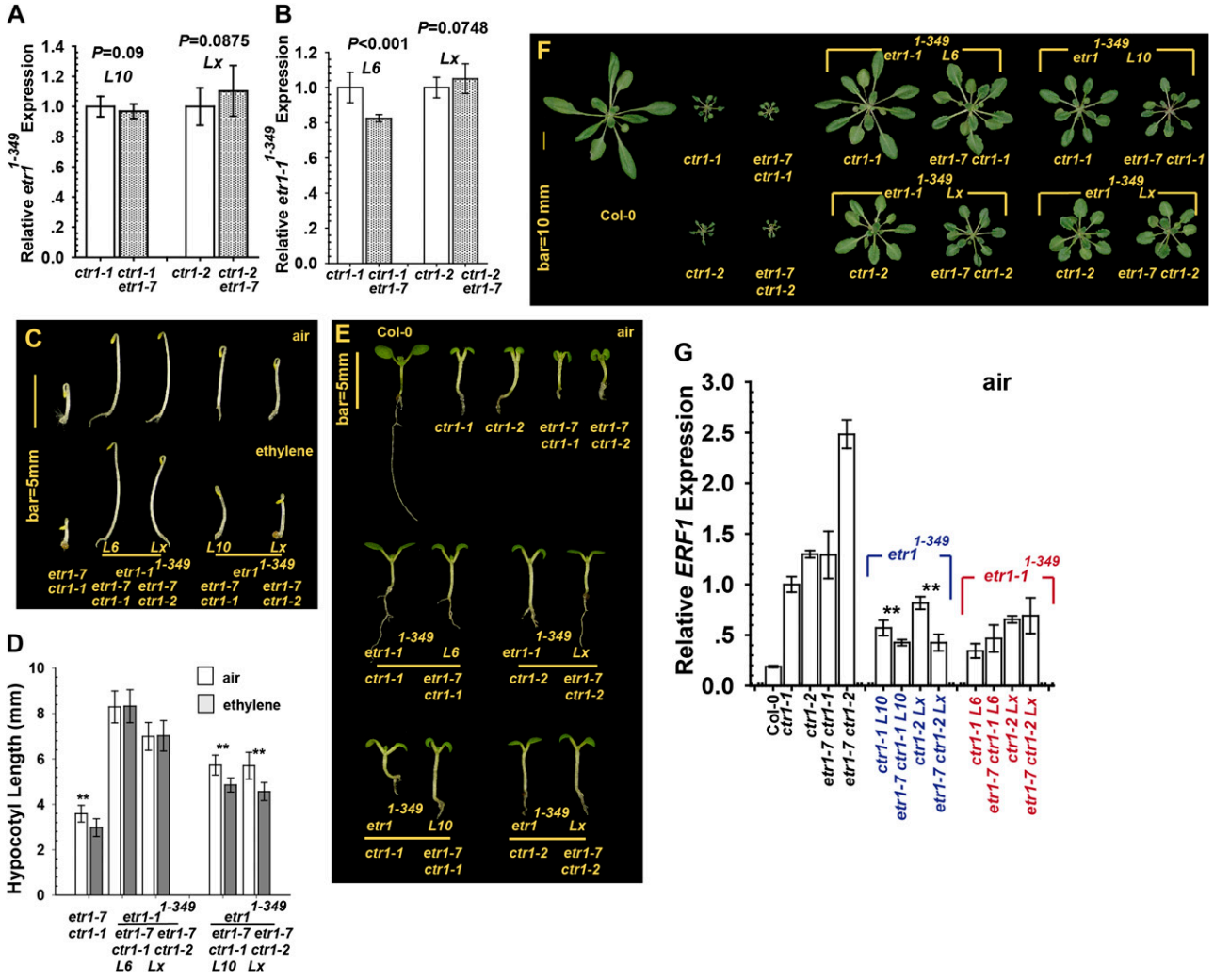

Figure 3. Growth rescue of *etr1 ctr1* mutants by the ETR1 N terminus. A and B, $etr1^{1\text{-}349}$ (A) and $etr1\text{-}1^{1\text{-}349}$ (B) expression in transgene donors (*ctr1-1* and *ctr1-2* lines) and recipients (*etr1-7 ctr1-1* and *etr1-7 ctr1-2* lines). C and D, Phenotype (C) and hypocotyl length (D) of etiolated *etr1-7 ctr1* seedlings expressing the $etr1\text{-}1^{1\text{-}349}$ or $etr1^{1\text{-}349}$ transgene. E and F, Phenotype of light-grown seedlings (E) and rosettes (F) of mutants expressing the $etr1\text{-}1^{1\text{-}349}$ or $etr1^{1\text{-}349}$ transgene. G, Relative *ERF1* expression level of transformation mutants. *L* indicates the transformation lines. Data are means ± SD. ** $P < 0.01$ by Student's *t* test. [See online article for color version of this figure.]

mutants showed larger and more expanded cotyledons and longer seedling hypocotyls and roots (Fig. 3E). At the adult stage, *etr1-7 ctr1-1* and *etr1-7 ctr1-2* plants showed severe growth inhibition and small rosettes. Expression of $etr1\text{-}1^{1\text{-}349}$ or $etr1^{1\text{-}349}$ substantially suppressed the growth defects of the two mutants. Lack of the wild-type ETR1 seemed to have a minor effect on suppression of the *ctr1* mutant phenotype by the transgenes, and *ctr1-1* and *ctr1-2* lines that expressed the ETR1 N terminus were slightly larger than corresponding *ctr1 etr1-7* transformation lines (Fig. 3F).

We evaluated the degree of constitutive ethylene response in transformation mutants by measuring *ERF1* expression. *ERF1* levels were higher (greater than 5-fold) in air-grown *ctr1-1*, *ctr1-2*, *etr1-7 ctr1-1*, and *etr1-7 ctr1-2* plants than in wild-type (Col-0) plants. *ERF1* levels were slightly higher in *ctr1* transformation lines (*L10* and *Lx*) with $etr1^{1\text{-}349}$ than in corresponding *etr1-7 ctr1* $etr1^{1\text{-}349}$ lines (Student's *t* test, $P < 0.01$). In contrast, *ERF1* expression was identical in *ctr1* $etr1\text{-}1^{1\text{-}349}$ transformation lines (*L6* and *Lx*) and corresponding *etr1-7 ctr1* $etr1\text{-}1^{1\text{-}349}$ transformation lines (Student's *t* test, $P > 0.05$; Fig. 3G).

The constitutive ethylene response of *ctr1-1* and *ctr1-2* suppressed by the ETR1 N terminus was not affected in the absence of ETR1. Given that the suppression of the constitutive ethylene response by $etr1\text{-}1^{1\text{-}349}$ predominantly depends on ETR1 and ERS1 and that the ethylene receptors cooperate differentially (Xie et al.,

2006; Gao et al., 2008; Liu and Wen, 2012), the ETR1 N terminus may cooperate differentially with other ethylene receptors to mediate the receptor signal independent of CTR1.

RTE1 Is Essential for Suppression of the *ctr1-1* Mutant Phenotype by etr1$^{1-349}$

RTE1 overexpression leads to ethylene insensitivity in wild-type (Col-0) plants but not in the *etr1-7* loss-of-function mutant, and expression of the *etr1*$^{1-349}$ transgene restores the ethylene insensitivity conferred by *RTE1* overexpression in *etr1-7* (Resnick et al., 2006; Zhou et al., 2007). The physical association of ETR1 and RTE1 plays a role in regulating ETR1 signaling (Dong et al., 2010). These studies indicate that excess RTE1 promotes ETR1 N-terminal signaling but do not conclude whether RTE1 is essential for ETR1 N-terminal signaling. We wondered whether the RTE1-promoted ETR1 N-terminal signaling could also be independent of CTR1. We addressed these questions by examining whether expression of the ETR1 N terminus still confers ethylene insensitivity both in the *ctr1-1* background when *RTE1* is overexpressed and in the *rte1-2 ctr1-1* loss-of-function mutant.

Air-grown etiolated *ctr1-1* seedlings showed the constitutive ethylene-response phenotype and produced a short seedling hypocotyl. *RTE1* overexpression (designated *RTE1ox*) did not alleviate the seedling growth inhibition of *ctr1-1*. In contrast, when *35S:RTE1* and *etr1*$^{1-349}$ were coexpressed, the constitutive ethylene-response phenotype was substantially suppressed in air-grown *ctr1-1* seedlings and ethylene treatment did not inhibit seedling growth (Fig. 4A). Seedling hypocotyl length was the same as in the seedling triple-response assay, with the hypocotyl elongation of *RTE1ox etr1*$^{1-349}$ *ctr1-1* seedlings unaffected by ethylene treatment (Student's *t* test, $P > 0.14$; Fig. 4B).

Light-grown *ctr1-1* seedlings showed a constitutive ethylene-response phenotype in air: the cotyledons were small, and the seedling hypocotyls and roots were short. The *ctr1-1* seedling growth defects were substantially suppressed by the *etr1*$^{1-349}$ transgene: *etr1*$^{1-349}$ *ctr1-1* (*L10*) showed larger and more expanded cotyledons, with longer seedling hypocotyls and roots than *ctr1-1*. As expected, *RTE1* overexpression had little effect on *ctr1-1* seedling growth but substantially promoted the growth of *etr1*$^{1-349}$ *ctr1-1* (*L10*) seedlings, which had much larger cotyledons and a longer hypocotyl and root than *etr1*$^{1-349}$ *ctr1-1* (*L10*) seedlings (Fig. 4C).

Grown to the adult stage, the *ctr1-1* mutant phenotype was in part suppressed by the *etr1*$^{1-349}$ transgene: rosettes were larger for *etr1*$^{1-349}$ *ctr1-1* (*L10*) than for *ctr1-1* plants. As expected, *RTE1* overexpression did not affect *ctr1-1* plant growth but substantially promoted the growth of *etr1*$^{1-349}$ *ctr1-1* (*L10*) plants, and rosettes were larger for *RTE1ox etr1*$^{1-349}$ *ctr1-1* (*L10*) than for *etr1*$^{1-349}$ *ctr1-1* (*L10*) plants (Fig. 4D).

Our data suggested that RTE1 overexpression promoted ETR1 N-terminal signaling in the absence of CTR1. We examined whether RTE1 is essential to the CTR1-independent ETR1 N-terminal signaling by examining whether the *rte1-2* allele reverses the *etr1*$^{1-349}$ *ctr1-1* phenotype. In the seedling triple-response assay, we showed that in the presence of the *etr1*$^{1-349}$ transgene, seedling hypocotyls were longer for *ctr1-1* than for *rte1-2 ctr1-1*, and ethylene treatment inhibited in part the hypocotyl elongation (Fig. 4, E and F). Hypocotyls were slightly longer for *ctr1-1* than for *rte1-2 ctr1-1* seedlings in air and ethylene (Student's *t* test, $P < 0.01$). Light-grown *rte1-2 ctr1-1* seedlings showed severe growth inhibition and had much smaller cotyledons and shorter hypocotyls than *ctr1-1* seedlings. Interestingly, the *ctr1-1* growth defects suppressed by *etr1*$^{1-349}$ were prevented by the *rte1-2* allele (Fig. 4G). Grown to the adult stage, rosettes were smaller for *rte1-2 etr1*$^{1-349}$ *ctr1-1* than for *ctr1-1* plants. As expected, rosettes were smaller for *rte1-2 ctr1-1* than for *ctr1-1* (Fig. 4H).

We quantitatively evaluated the degree of the constitutive ethylene response of *ctr1-1* altered by the expression of *35S:RTE1* and *etr1*$^{1-349}$. With the *ERF1* expression of ethylene-treated *ctr1-1* plants set to 1, the level of *ERF1* was not altered in *RTE1ox ctr1-1*, whereas that of *etr1*$^{1-349}$ *RTE1ox ctr1-1* was substantially attenuated (Fisher's LSD, $\alpha = 0.01$; Fig. 4I). We next examined the expression of *CHIB* and *PDF1.2*, whose expression is elevated by ethylene treatment (Hua and Meyerowitz, 1998). As expected, the levels of both *CHIB* and *PDF1.2* in *ctr1-1* were similar to that in *RTE1ox ctr1-1* and were highly attenuated in *etr1*$^{1-349}$ *RTE1ox ctr1-1* (Fisher's LSD, $\alpha = 0.01$; Fig. 4, J and K). qRT-PCR revealed the expression of the *etr1*$^{1-349}$ transgene to be identical in the transgene donor (*etr1*$^{1-349}$ *ctr1-1*, *L10*) and the recipient (*RTE1ox etr1*$^{1-349}$ *ctr1-1*; Student's *t* test, $P > 0.25$; Fig. 4L).

Our data here indicate that RTE1 was essential to the CTR1-independent ETR1 N-terminal signaling, which suppresses constitutive ethylene responses. The stronger growth defects of *etr1-7 ctr1-1* and *rte1-2 ctr1-1* than of *ctr1-1* agree with the suggestion that RTE1 promotes a portion of ETR1 signaling that is independent of CTR1, so that the lack of ETR1 or RTE1 will strengthen the *ctr1-1* mutant phenotype.

The Constitutive Ethylene-Response Phenotype of *CTR1-Nox* Is Suppressed by *etr1*$^{1-349}$ and *etr1-1*$^{1-349}$

Our results suggested that mediation of ETR1 N-terminal signaling is independent of CTR1. Given that the ethylene receptors act cooperatively and that the ETR1 N-terminal signaling primarily depends on ETR1 and ERS1, ETR1 N-terminal signaling to full-length ethylene receptors may be mediated by a pathway independent of CTR1. Alternatively, the signal output of full-length ethylene receptors may be cooperatively mediated to the ETR1 N terminus via a

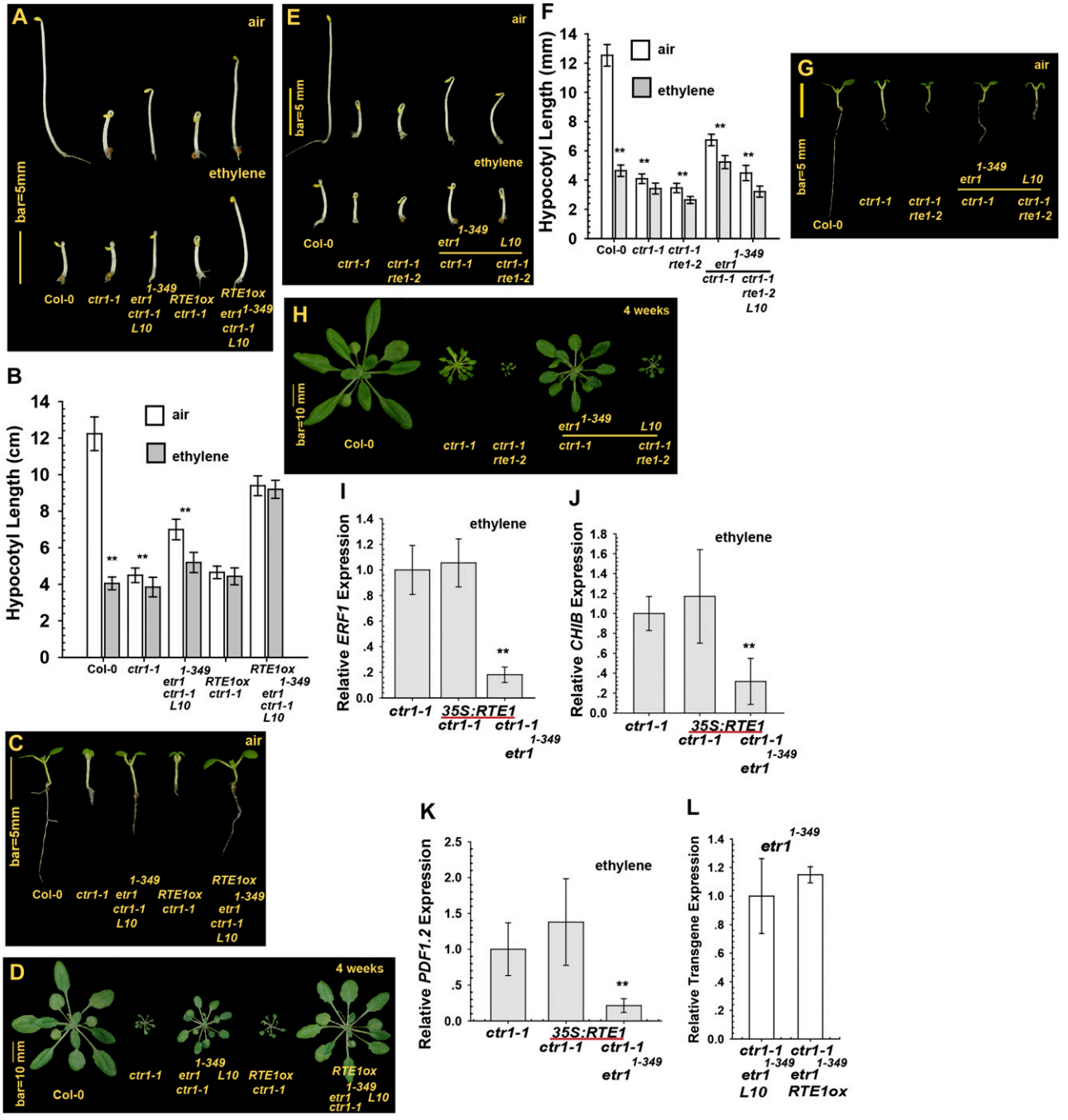

Figure 4. *RTE1* is required for the rescue of *ctr1-1* growth by *etr1*$^{1-349}$. A and B, Phenotype (A) and hypocotyl length (B) of etiolated *ctr1-1* seedlings with or without the *etr1*$^{1-349}$ and *35S:RTE1* transgenes. C and D, Phenotype of light-grown *ctr1-1* seedlings (C) and adult plants (D) with or without the *etr1*$^{1-349}$ and *35S:RTE1* transgenes. E and F, Phenotype (E) and seedling hypocotyl length (F) of *ctr1-1*, *ctr1-1 rte1-2*, and mutants with the *etr1*$^{1-349}$ transgene. G and H, Phenotype of light-grown seedlings (G) and rosettes (H) of *ctr1-1*, *ctr1-1 rte1-2*, and mutants with the *etr1*$^{1-349}$ transgene. I to K, Relative expression of *ERF1* (I), *CHIB* (J), and *PDF1.2* (K) in *ctr1-1* with or without *etr1*$^{1-349}$ and *35S:RTE1* transgenes. L, Relative expression of the *etr1*$^{1-349}$ transgene in *etr1*$^{1-349}$ *ctr1-1* and *RTE1ox etr1*$^{1-349}$ *ctr1-1*. *L10*, The *etr1*$^{1-349}$ transgene was from the transformation line *etr1*$^{1-349}$*ctr1-1 L10* by genetic cross. Data are means ± SD. ** $P < 0.01$ by Student's *t* test (B) and Fisher's LSD (G–I). [See online article for color version of this figure.]

CTR1-indpendent pathway (Fig. 5A; Gamble et al., 2002; Xie et al., 2006; Gao et al., 2008; Gao and Schaller, 2009; Chen et al., 2010; Liu and Wen, 2012). Given that excess CTR1$^{7\text{-}560}$ prevents ethylene receptor signaling (Huang et al., 2003), if the constitutive ethylene-response phenotype of *CTR1-Nox* is suppressed by expression of the ETR1 N terminus, ETR1 N-terminal signaling may not be mediated via full-length ethylene receptors.

We transformed *35S:CTR1*$^{7\text{-}560}$ into the wild type (Col-0) and selected three transformation lines (designated *CTR1-Nox*) representing three categories of constitutive ethylene response. Seedling triple-response phenotype and hypocotyl length data showed *CTR1-Nox* (*L50*) with the strongest constitutive ethylene-response phenotype and *L63* with the weakest (Fig. 5, B and C). Consistently, growth was inhibited in light-grown seedlings of the three lines, which showed small cotyledons and short hypocotyls and roots, with *L50* showing the greatest growth inhibition and *L63* the least (Fig. 5D). Immunoassay revealed an association of the expression of the CTR1$^{7\text{-}560}$ fragment and the degree of the constitutive ethylene-response phenotype, with *L50* showing the highest CTR1$^{7\text{-}560}$ expression and *L63* the least (Fig. 5E).

We introduced the *etr1-1*$^{1\text{-}349}$ and *etr1*$^{1\text{-}349}$ transgenes from transgene donors *L6* and *L10*, respectively, by genetic crossing (Fig. 1), into *CTR1-Nox* (*L50*). As expected, the seedling hypocotyl length of *CTR1-Nox* (*L50*) was longer with than without the transgenes,

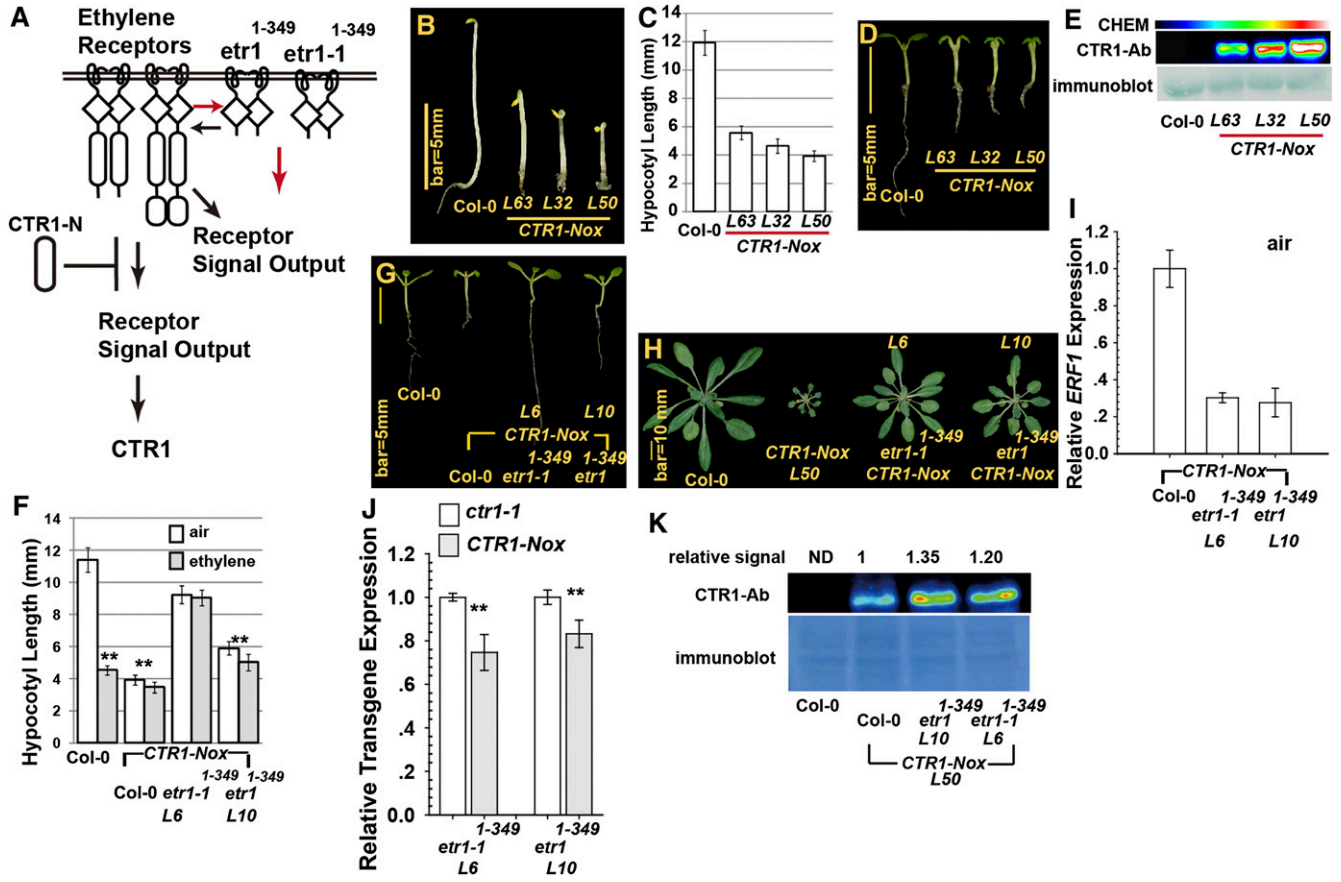

Figure 5. Expression of the ETR1 N terminus suppresses the *CTR1-Nox* constitutive ethylene response. A, Scenarios of the ETR1 N-terminal signal mediated by full-length ethylene receptors (black arrows) or full-length ethylene receptor signals mediated by the ETR1 N terminus (red arrows) by an alternative pathway when excess CTR1$^{7\text{-}560}$ prevents ethylene receptor signaling. B to D, Phenotype (B), hypocotyl length (C), and seedling growth (D) of wild type (Col-0) plants expressing *35S:CTR1*$^{7\text{-}560}$ (designated *CTR1-Nox*). E, Immunoassay of expression of the CTR1$^{7\text{-}560}$ fragment in *CTR1-Nox* lines. F to H, Hypocotyl length (F), seedling growth (G), and rosette phenotype (H) of *CTR1-Nox L50* expressing *etr1-1*$^{1\text{-}349}$ or *etr1*$^{1\text{-}349}$. I, Relative *ERF1* expression in *CTR1-Nox* and *CTR1-Nox* expressing the ETR1 N terminus. J, Relative transgene expression in the transgene donor (Col-0) and recipient (*CTR1-Nox*). K, Immunoassay of CTR1$^{7\text{-}560}$ expression. *L6* and *L10* indicate the origins of the *etr1-1*$^{1\text{-}349}$ and *etr1*$^{1\text{-}349}$ transformation lines, respectively. CHEM, The pseudocolor bar indicates relative chemiluminescence strength from weak (dark) to strong (bright); CTR1-Ab, chemiluminescence detected by the monoclonal CTR1 antibody; Immunoblot, staining with Coomassie blue to show relative protein amount; ND, not determined. Data are means ± SD. ** $P < 0.01$ by Student's *t* test. [See online article for color version of this figure.]

which indicates that the growth inhibition of *CTR1-Nox* (*L50*) was prevented in part by each transgene, with *etr1-1*$^{1-349}$ having a greater effect than *etr1*$^{1-349}$. Ethylene treatment did not affect the growth of *etr1-1*$^{1-349}$ *CTR1-Nox*, and the hypocotyl lengths of air- and ethylene-grown *etr1-1*$^{1-349}$ *CTR1-Nox* (*L50*) seedlings were identical (Student's *t* test, $P > 0.29$). As expected, the elongation of *etr1*$^{1-349}$ *CTR1-Nox* (*L50*) seedlings was inhibited by ethylene treatment (Student's *t* test, $P < 0.01$; Fig. 5F). Grown under light, *CTR1-Nox* (*L50*) seedlings showed severe growth inhibition and produced small cotyledons and short hypocotyls and roots. The expression of *etr1-1*$^{1-349}$ and *etr1*$^{1-349}$ substantially suppressed the growth defects: the *CTR1-Nox* seedlings carrying the transgene showed expanded cotyledons and produced long hypocotyls and roots (Fig. 5G). At the adult stage, the strong rosette growth inhibition of *CTR1-Nox* was substantially prevented by the expression of each transgene (Fig. 5H).

We evaluated the degree of *CTR1-Nox* (*L50*) ethylene response altered by the etr1 isoforms by measuring *ERF1* expression. qRT-PCR revealed that the expression of *etr1-1*$^{1-349}$ and *etr1*$^{1-349}$ substantially attenuated *ERF1* expression in *CTR1-Nox* (*L50*) by about 70% (Fig. 5I). As well, levels of transgenes were slightly lower in *CTR1-Nox* than in corresponding donors (Fig. 5J). Transgene attenuation of *ERF1* expression and prevention of the constitutive ethylene-response phenotype of *CTR1-Nox* were unlikely to be affected by minor alterations in transgene expression. We used an immunoassay to examine whether the truncated etr1 isoform recovery of growth and attenuation of *ERF1* expression in *CTR1-Nox* resulted from impaired CTR1$^{7-560}$ expression. CTR1$^{7-560}$ expression in the two transformation lines was not reduced as compared with that of the *35S:CTR1*$^{7-560}$ donor [*CTR1-Nox* (*L50*); Fig. 5K].

The constitutive ethylene-response phenotype of *CTR1-Nox* was prevented in part by expression of the ETR1 N terminus, so the ETR1 N-terminal signaling was not mediated by full-length ethylene receptors. Rather, full-length ethylene receptor signaling likely occurs cooperatively via the ETR1 N terminus through a pathway independent of CTR1.

Expression of *etr1*$^{1-349}$ Suppresses the Constitutive Ethylene-Response Phenotype and Confers Ethylene Insensitivity in *ctr1-1* Harboring an Ethylene-Insensitive Ethylene Receptor Gene

The signal output of full-length ethylene receptors via the ETR1 N terminus may be mediated by a pathway independent of CTR1 (Fig. 5A). Double mutants that carry *ctr1-1* and a dominant ethylene-insensitive receptor gene show the constitutive ethylene-response phenotype. If expression of the ethylene-responsive etr1$^{1-349}$ restores the ethylene insensitivity conferred by the ethylene-insensitive receptor gene, the ETR1 N terminus should mediate the signal output of full-length ethylene receptors by a pathway independent of CTR1.

etr1-1, *ers1-1*, *etr2-1*, *ein4-1*, and *ers2-1* are dominant ethylene receptor gene mutations, and each confers ethylene insensitivity. Ethylene treatment had little effect on their seedling growth, as seen by seedling hypocotyl length (Fig. 6A). Consistent with previous genetic analyses, the *ctr1-1* mutation suppressed each of the dominant, ethylene-insensitive receptor gene mutations, and the corresponding double mutants showed inhibited seedling growth regardless of ethylene treatment (Fig. 6B). In each of these double mutants, ectopic expression of *etr1*$^{1-349}$ suppressed the growth inhibition to various degrees, with or without ethylene treatment (Student's *t* test, $P < 0.01$; Fig. 6C). Among the double mutants, *etr1*$^{1-349}$ had the greatest effect on promoting the growth of *ers1-1 ctr1-1* and *ers2-1 ctr1-1*. Light-grown *ctr1-1* seedlings showed small and compact cotyledons and short hypocotyls and roots. Seedlings of these ethylene-insensitive receptor mutants showed large, expanded cotyledons and long hypocotyls and roots (Fig. 6D). As expected, growth was inhibited in these light-grown double-mutant seedlings, and double mutants carrying *etr1*$^{1-349}$ showed normal seedling growth, with large, extended cotyledons and long hypocotyls and roots (Fig. 6E). At the adult stage, the double mutants produced small rosettes; *etr1*$^{1-349}$ expression promoted the growth of these double mutants to a large extent. Among these mutants, *etr1*$^{1-349}$ *ctr1-1* carrying *ers-1* or *ers2-1* had the largest rosettes, and that carrying *ein4-1* had the smallest (Fig. 6F).

In addition to measuring growth phenotype alterations, we examined *ERF1* expression by qRT-PCR to evaluate quantitatively the degree of ethylene responsiveness attenuated by the expression of *etr1*$^{1-349}$ in each double mutant. *ERF1* levels in ethylene-insensitive receptor mutants were only 2% to 5% that of *ctr1-1* levels (Fig. 6G). In *ctr1-1*, the *ERF1* level was attenuated to different levels with each of the ethylene-insensitive receptor gene mutations, except for *ers1-1* (Fig. 6H), which is in line with the *ctr1-1* mutation not completely suppressing each of the ethylene-insensitive receptor mutations (Fig. 6, B and E). As expected, *ERF1* levels were highly attenuated with *etr1*$^{1-349}$ expression in the double mutants (3.6%–12% that of *ctr1-1* levels; Fig. 6I). qRT-PCR analysis of *etr1*$^{1-349}$ transgene expression showed *etr1*$^{1-349}$ expression attenuated by about 20% to 45% that of the transgene donor (*etr1*$^{1-349}$ *ctr1-1 L10*). Therefore, the prevention of ethylene responses by *etr1*$^{1-349}$ expression in the double mutants was not due to elevated *etr1*$^{1-349}$ expression (Fig. 6, J and K).

Coexpression of *etr1*$^{1-349}$ and an ethylene-insensitive receptor gene substantially suppressed the *ctr1-1* mutant phenotype and conferred ethylene insensitivity. Our results support that the signal of each of the five ethylene receptors can be cooperatively mediated via the ETR1 N terminus to suppress the constitutive ethylene response via a pathway independent of CTR1.

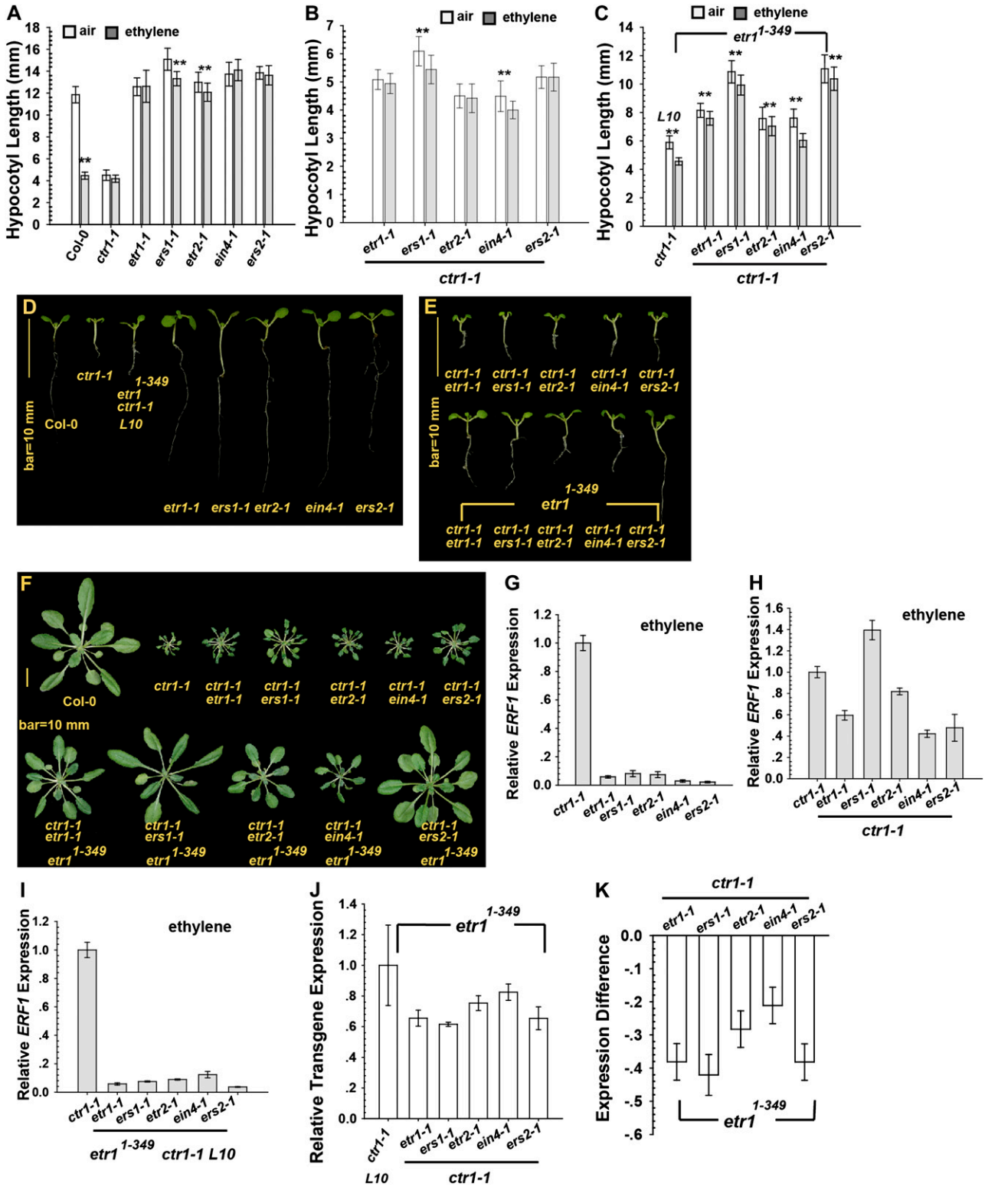

Figure 6. Expression of *etr1*$^{1-349}$ restores ethylene insensitivity conferred by dominant receptor mutant alleles in the *ctr1-1* background. A to C, Seedling hypocotyl length of *ctr1-1* and ethylene-insensitive receptor mutants (A), double mutants of *ctr1-1* with an ethylene-insensitive receptor mutation (B), and double mutants expressing *etr1*$^{1-349}$ (C). D, Phenotype of light-grown seedlings of *ctr1-1* and ethylene-insensitive receptor mutants. E and F, Phenotype of double mutants of *ctr1-1* with an ethylene-insensitive receptor mutation, with and without the *etr1*$^{1-349}$ transgene, at seedling (E) and rosette (F) stages. G to I, Relative *ERF1* expression in *ctr1-1* and each ethylene-insensitive receptor mutant (G), *ctr1-1* with an ethylene-insensitive receptor mutation

DISCUSSION

Previous studies suggested that CTR1 acts downstream of ethylene receptors and that the ETR1-CTR1 association mediates the ethylene receptor signal output. Notably, *ctr1* mutants show a constitutive ethylene-response phenotype but are somewhat ethylene responsive. The transcription factors EIN3 and EIN3-Like1 (EIL1) up-regulate the expression of ethylene-inducible genes. The constitutive ethylene-response phenotype is stronger in mutants defective in multiple ethylene receptors or the F-box proteins EBF1 and EBF2, which mediate EIN3/EIL1 degradation, than in *ctr1* mutants (Kieber et al., 1993; Chao et al., 1997; Clark et al., 1998; Hua and Meyerowitz, 1998; Cancel and Larsen, 2002; Gao et al., 2003; Guo and Ecker, 2003; Huang et al., 2003; Potuschak et al., 2003). Therefore, a portion of the ethylene receptor signal may be mediated independently of CTR1, whereby lack of CTR1 substantially weakens but does not abolish the ethylene receptor signal output. Interestingly, we found that without CTR1, the ETR1 N terminus could produce the ethylene receptor signal output to suppress the constitutive ethylene response, which supports the hypothesis that the ethylene receptor signal can be mediated by a pathway independent of CTR1.

The ETR1 N terminus consists of three transmembrane helices and the GAF domain, of which the transmembrane helices are the ethylene-binding site and the GAF domain is involved in noncovalent receptor dimerization. ETR1 N-terminal signaling primarily depends on ETR1 and ERS1, which suggests cooperative receptor signaling of full-length ethylene receptors with the ETR1 N-terminal fragment (Rodríguez et al., 1999; Wang et al., 2006; Xie et al., 2006; Gao et al., 2008). The cooperative signaling could occur via mediation of the ETR1 N-terminal signal to full-length ethylene receptors or the opposite way, to a pathway independent of CTR1 (Fig. 5A; Gamble et al., 2002; Xie et al., 2006). We found the constitutive ethylene-response phenotype of *CTR1-Nox*, with the receptor signaling prevented by excess CTR1$^{7\text{-}560}$, suppressed by expression of the ETR1 N terminus. These results do not favor the scenario of the ETR1 N-terminal signal output mediated by full-length ethylene receptors. Rather, the favored scenario is that the full-length ethylene receptor signal is mediated cooperatively via the ETR1 N terminus by a pathway independent of CTR1. This hypothesis is also supported by results showing that expression of *etr1*$^{1\text{-}349}$ suppressed the constitutive ethylene-response phenotype and conferred ethylene insensitivity in double mutants harboring *ctr1-1* and an ethylene-insensitive receptor allele. Given that the ETR1 N-terminal GAF domain can form heterodimers with other ethylene receptors (Xie et al., 2006; Gao et al., 2008), the GAF domain may have a role in interreceptor signaling and may be where the N-terminal signal output occurs.

The components that mediate or modulate the ETR1 N-terminal signal have yet to be identified. RTE1 overexpression promotes ETR1 N-terminal signaling in *etr1-7* (Zhou et al., 2007) and ETR1 and RTE1 physically associate (Dong et al., 2010), so RTE1 may be involved in the CTR1-independent ETR1 N-terminal signaling. This hypothesis was supported by our results showing that expression of *etr1*$^{1\text{-}349}$ did not suppress the *ctr1-1* mutant phenotype in the presence of the *rte1-2* loss-of-function mutation and that *RTE1* overexpression conferred ethylene insensitivity and suppressed the constitutive ethylene-response phenotype in the *ctr1-1* mutant expressing the *etr1*$^{1\text{-}349}$ transgene. These results also reveal an essential role of RTE1 in ETR1 N-terminal signaling.

We observed CTR1-independent ETR1 N-terminal signaling only in the presence of the *etr1*$^{1\text{-}349}$ or *etr1-1*$^{1\text{-}349}$ transgene, which encodes the truncated ETR1 protein lacking the C terminus. Therefore, ETR1 N-terminal signaling mediated by the native, full-length ETR1 was largely prevented in *ctr1* mutants. The ETR1 C terminus may inhibit N-terminal signaling so that the N-terminal signaling mediated by the full-length ETR1 did not occur in *ctr1* mutants or *ctr1-1* carrying *35S*:*RTE1* or an ethylene-insensitive receptor allele. In contrast, deletion of the ETR1 C terminus alleviated the signaling inhibition, thereby activating ETR1 N-terminal signaling. The C terminus could directly prevent N-terminal signaling or disrupt the ethylene receptor cooperativity, which is essential to N-terminal signaling. Given that the full-length ETR1 ethylene receptor has the C-terminal portion, if the ETR1 C terminus could disrupt receptor cooperativity, it would also disrupt the cooperativity of the full-length ETR1 and the ETR1 N terminus and disrupt signaling. Thus, we do not favor the scenario that the ETR1 C terminus disrupts ethylene receptor cooperativity with the ETR1 N terminus.

The CTR1 N terminus associates physically with the ETR1 C-terminal HK domain, which mediates the ethylene receptor signal. CTR1 isoforms with mutations in the C-terminal kinase domain, such as ctr1-1 and ctr1-4, may associate with ethylene receptors on the endoplasmic reticulum (Gao et al., 2003), so CTR1 kinase activity is essential to ethylene receptor signaling. This argument is in agreement with the result showing that an excess amount of CTR1$^{7\text{-}560}$, which does not have a CTR1 kinase domain, prevents

Figure 6. (*Continued.*)
(H), and double mutants expressing etr1$^{1\text{-}349}$(I). J, Relative etr1$^{1\text{-}349}$ transgene expression in the transgene donor (ctr1-1 L10) and recipients (the double mutants). K, Difference in expression (by Fisher's LSD) of the etr1$^{1\text{-}349}$ transgene for double mutants and transgene donor. Data are means ± SD. ** $P < 0.01$ by Student's t test. [See online article for color version of this figure.]

ethylene receptor signaling, possibly by titrating out available ethylene receptors (Huang et al., 2003). The suppression of the *CTR1-Nox* phenotype by expression of *etr1*$^{1-349}$ and *etr1-1*$^{1-349}$, the latter of which also conferred ethylene insensitivity, indicated that CTR1$^{7-560}$ did not physically affect ETR1 N-terminal functions in cooperation with full-length ethylene receptors. Another implication is that CTR1 kinase activity may modulate ETR1 N-terminal signaling, in addition to its role in mediating ethylene receptor signaling. CTR1 kinase activity may inhibit the ETR1 C-terminal inhibition with the ETR1-CTR1 association, thereby facilitating ETR1 N-terminal signaling. The association of a kinase-defective CTR1 isoform with ETR1 fails to alleviate the ETR1 C-terminal inhibition, and the ETR1 N-terminal signaling is not facilitated. This argument may explain the lack of ETR1 N-terminal signaling in *ctr1* mutants with defective kinase activity.

In summary, we hypothesize that modulation of the ethylene receptor ETR1 signal output involves CTR1, RTE1, and possibly ethylene receptor cooperation and negative regulation by the ETR1 C terminus. The receptor signal of full-length ETR1 can be mediated via CTR1 and a CTR1-independent pathway. The ethylene receptor signaling mediated via CTR1 has been well addressed in previous studies. Results from our study suggest that the ETR1 N terminus plays important roles in ethylene receptor signaling via a CTR1-independent pathway. The ETR1 C terminus may inhibit the ETR1 N-terminal signaling. Deletion of the C terminus or the association of CTR1 with the ETR1 C terminus alleviates the ETR1 C-terminal negative regulation. CTR1 kinase activity may have a role in eliminating the C-terminal inhibition. Without the ETR1 C-terminal inhibition, RTE1 promotes the ETR1 N-terminal signaling. The GAF domain may involve receptor interaction, by which receptor cooperation occurs and the signal of full-length ethylene receptors is mediated via CTR1 and the ETR1 N terminus, with the latter independent of CTR1, to repress ethylene responses.

MATERIALS AND METHODS

Plant Materials and Transgenes

Arabidopsis (*Arabidopsis thaliana*) *etr1-1*, *ers1-1*, *etr2-1*, *ein4-1*, and *ers2-1* were each genetically crossed with *ctr1-1* to obtain double mutants. *etr1-7* was crossed with *ctr1-1* or *ctr1-2* to obtain double mutants. Receptor-defective mutants were described previously (Xie et al., 2006; Zhou et al., 2007). Corresponding transgenes and transformation lines carrying *35S:RTE1*, *ETR1p:etr1*$^{1-349}$, and *ETR1p:etr1-1*$^{1-349}$ were described previously (Xie et al., 2006; Zhou et al., 2007). *ctr1-2* was obtained from J. Kieber (Huang et al., 2003). To clone the CTR1$^{7-560}$ -encoding fragment, a full-length genomic CTR1 fragment, restricted with *Eco*RV and *Sma*I, was cloned from the bacterial artificial chromosome clone *F17C15* to pBluescript SK+. The primer set CTR1 N1-F (5′-CGCTCGAGATGAGATCTAATTACACTTTGC-3′) and CTR1 N1-R (5′-AGGTCGATGCATAAGGT-3′) amplified a 5′ fragment carrying the start codon (ATG). The primer set CTR1 N2-F (5′-CCGGCTTAATGAATTCTAG-TGC-3′) and CTR1 N2-R (5′-CAACTAGTTTAACCTGAAAGAGCAGTCG-CTCAA-3′) amplified a 3′ fragment carrying a stop codon (TAA). The 5′ ATG-containing fragment was swapped with the *Xho*I/*Nsi*I fragment of the genomic *CTR1* clone; the 3′ TAA-containing fragment was swapped with the *Eco*RI/*Spe*I fragment of the genomic *CTR1* clone, of which the *Spe*I site was from the cloning vector. The resulting genomic fragment, encoding CTR1$^{7-560}$, was released with *Kpn*I and *Xba*I and cloned to the cauliflower mosaic virus *35S* promoter-containing binary vector pCAMBIA. Phenotypic analyses of these transformation lines were carried out in the F4, or more advanced, generations.

Ethylene Treatment and Seedling Hypocotyl Measurement

Ethylene treatment and measurement were as described (Zhang and Wen, 2010). Ethylene (10 μL L^{-1}) was used in the seedling triple-response assay and gene expression analyses. For each hypocotyl measurement, at least 20 seedlings ($n \geq 20$) were measured (Xie et al., 2006). For gene expression analyses, adult plants (4 weeks old) were treated with ethylene for 4 h and then subjected to qRT-PCR analysis.

Immunoassay

An IgG monoclonal antibody purified from mice ascites against the CTR1$^{1-452}$ fragment was prepared by an antibody service (Abmart). Total protein from *35S:CTR1*$^{7-560}$ transformation lines was isolated and fractionated by SDS-PAGE (Wen et al., 1999; Liu et al., 2010). The protein was transferred to a polyvinylidene difluoride membrane and probed with the monoclonal antibody. The monoclonal IgG was next probed with a horseradish peroxidase-conjugated goat anti-mouse IgG and detected by enhanced chemiluminescence captured with the use of a cold (−110°C) CCD system (VersArray System; Princeton Instruments; Liu et al., 2010) and computed by the use of MetaMorph version 7.0 (Molecular Devices). The same immunoblot was stained with Coomassie blue to visualize relative protein amount on membranes.

Fluorescence qRT-PCR

qRT-PCR and primer sets were described previously (Liu et al., 2010; Zhang and Wen, 2010). qRT-PCR involved the use of the StepOnePlus system (ABI). Primers used were PDF1.2 F (5′-CTTTGCTGCTTTCGACGCACC-3′) and PDF1.2 R (5′-CATGGGACGTAACAGATACA-3′); etr1-1F (5′-GCTTT-TATCGTTCTTTA-3′) and etr1-1R (5′-GCTTTATTTTTCAAGAAA-3′); and ETR1N-F (5′-CGCTGATCAGGTGGCTGTAG-3′) and ETR1N-R (5′-TCTAG-AGGATCCTAAACCGC-3′). Each measurement was repeated three times with three independent biological replicates.

Statistical Analysis

Data were compared by Student's *t* test or, for paired comparison of multiple means and estimation of the mean difference, Fisher's LSD, with $P < 0.05$ or $P < 0.01$ indicating statistical significance.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NC_003070, NC_003071, NC_003074, NC_003074, NC_003070, NC_003071, and NC_003076.

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