Arabidopsis *ETR1* and *ERS1* Differentially Repress the Ethylene Response in Combination with Other Ethylene Receptor Genes[1][W]

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The ethylene response is negatively regulated by a family of five ethylene receptor genes in Arabidopsis (*Arabidopsis thaliana*). The five members of the ethylene receptor family can physically interact and form complexes, which implies that cooperativity for signaling may exist among the receptors. The ethylene receptor gene mutations *etr1-1*$^{(C65Y)}$(for *ethylene response1-1*), *ers1-1*$^{(I62P)}$ (for *ethylene response sensor1-1*), and *ers1*^{C65Y} are dominant, and each confers ethylene insensitivity. In this study, the repression of the ethylene response by these dominant mutant receptor genes was examined in receptor-defective mutants to investigate the functional significance of receptor cooperativity in ethylene signaling. We showed that *etr1-1*$^{(C65Y)}$, but not *ers1-1*$^{(I62P)}$, substantially repressed various ethylene responses independent of other receptor genes. In contrast, wild-type receptor genes differentially supported the repression of ethylene responses by *ers1-1*$^{(I62P)}$; *ETR1* and *ETHYLENE INSENSITIVE4* (*EIN4*) supported *ers1-1*$^{(I62P)}$ functions to a greater extent than did *ERS2*, *ETR2*, and *ERS1*. The lack of both *ETR1* and *EIN4* almost abolished the repression of ethylene responses by *ers1*^{C65Y}, which implied that ETR1 and EIN4 have synergistic effects on ers1^{C65Y} functions. Our data indicated that a dominant ethylene-insensitive receptor differentially repressed ethylene responses when coupled with a wild-type ethylene receptor, which supported the hypothesis that the formation of a variety of receptor complexes may facilitate differential receptor signal output, by which ethylene responses can be repressed to different extents. We hypothesize that plants can respond to a broad ethylene concentration range and exhibit tissue-specific ethylene responsiveness with differential cooperation of the multiple ethylene receptors.

Ethylene is the first identified gaseous phytohormone and regulates aspects of many developmental processes in plants (Penmetsa and Cook, 1997; Ciardi et al., 2000; Block et al., 2005; Xu et al., 2006; Kevany et al., 2007; Boualem et al., 2009; Hattori et al., 2009). Arabidopsis (*Arabidopsis thaliana*) has five ethylene receptors, ETHYLENE RESPONSE1 (ETR1), ETR2, ETHYLENE RESPONSE SENSOR1 (ERS1), ERS2, and ETHYLENE INSENSITIVE4 (EIN4), which transmit a signal to the downstream kinase Constitutive Triple Response1 (CTR1) by a mechanism that has yet to be fully elucidated (Chang et al., 1993; Hua et al., 1995, 1998; Wang et al., 2003). Air-grown mutants defective in multiple receptors display many aspects of the constitutive ethylene response, which is indicative of redundancy and negative regulation of the receptor genes in ethylene signaling (Hua and Meyerowitz, 1998).

Duplicated loci may undergo subfunctionalization so that they can be stabilized in the genome (Thomas, 1993; Nowak et al., 1997). Subfunctionalization of ethylene receptors has been demonstrated in previous studies. ETR1 has a role in the seedling nutation and specifically acts with the Golgi/endoplasmic reticulum protein Reversion-to-Ethylene Sensitive1. ETR2 regulates trichome branching, and ERS1 negatively modulates ethylene receptor signaling in an ETR1-dependent manner (Binder et al., 2006; Zhou et al., 2007; Plett et al., 2009; Dong et al., 2010; Liu et al., 2010). The single loss-of-function mutants *etr1* and *ein4-7* both display hypersensitivity to ethylene, whereas *etr2* and *ers2* do not (Hua and Meyerowitz, 1998; Cancel and Larsen, 2002), which implies that *ETR1* and *EIN4* have unique roles in ethylene signaling. A synergistic (or superadditive) phenotype has been shown for *etr1 ers1-2* and *etr1 ers1-3* loss-of-function mutants, in which the double mutations result in extremely strong growth inhibition that exceeds the additive effects of the *etr1* and *ers1* mutations (Gao et al., 2003; Wang et al., 2003; Xie et al., 2006; Qu et al., 2007). Although the receptor genes are genetically redundant, their functions in ethylene signaling appear to be irreplaceable by each other (Wang et al., 2003; Klee, 2004; O'Malley et al., 2005; Liu et al., 2010).

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These results indicate that each member of the ethylene receptor family may have unique roles in ethylene signaling and may act cooperatively, rather than independently. This hypothesis is in accordance with results that show that the ETR1 receptor can physically dimerize with other members of the ethylene receptor family via noncovalent interactions, possibly via the GAF domain (Binder and Bleecker, 2003; Xie et al., 2006; Gao et al., 2008; Grefen et al., 2008; Gao and Schaller, 2009). Results from a membrane recruitment assay, with transient expression of fluorescence protein-tagged ethylene receptors in *Nicotiana benthamiana* epidermal cells, also suggest the formation of homomeric and heteromeric ethylene receptor protein complexes in vivo (Grefen et al., 2008). Gel-filtration analysis demonstrates that the ethylene receptors exist as components of high-molecular-weight protein complexes. The ethylene-induced molecular weight shift of the ERS1, but not ETR1, protein complex may indicate a unique regulation of the downstream ethylene response by ETR1 and ERS1 (Chen et al., 2010).

Ethylene receptor proteins are structurally similar to His kinase proteins of the "two-component" module that is prevalent in prokaryotes (Chang et al., 1993). In vitro assays demonstrated the His kinase activity of ETR1 and that the activity is inhibited by ethylene binding. The other receptors show Ser/Thr kinase activity (Gamble et al., 1998; Moussatche and Klee, 2004; Voet-van-Vormizeele and Groth, 2008). Mutational inactivation of ETR1 His kinase activity does not abolish ETR1's ability to repress ethylene responses (Gamble et al., 2002; Wang et al., 2003; Binder et al., 2004; Xie et al., 2006; Zhou et al., 2007; Kim et al., 2011), which indicates a lack of functional significance of His kinase activity in ethylene signaling. The lack of knowledge of the biochemical nature of the receptor signal limits advances in understanding the underlying molecular and biochemical bases of the receptor-mediated ethylene signal transduction. Nevertheless, analyses of alterations in various ethylene response phenotypes and the expression of ethylene-inducible genes have been used to comprehensively score the ethylene response qualitatively and quantitatively (Hua and Meyerowitz, 1998; Solano et al., 1998; Vogel et al., 1998; Gao et al., 2003; Qu and Schaller, 2004; Liu et al., 2010; Zhang and Wen, 2010).

The dominant *etr1-1* ethylene receptor gene mutation, causing the C65Y substitution, abolishes Cu(I)-mediated ethylene binding and confers ethylene insensitivity (Rodríguez et al., 1999). Whether the C65Y substitution converts ERS1 to a dominant, ethylene-insensitive isoform has yet to be investigated. The *etr1-4* mutation, which causes the I62F substitution, is also dominant, prevents ethylene binding, and results in ethylene insensitivity (Wang et al., 2006). In addition, *ers1-1* is an artificially created mutation that causes the I62P substitution and confers ethylene insensitivity (Hua et al., 1995).

Although previous studies lend strong support to the model that members of the ethylene receptor family act cooperatively as complexes, the functional significance of receptor cooperativity in the regulation of different ethylene responses has yet to be fully addressed. The reason why a plant would utilize multiple ethylene receptor genes to modulate ethylene responses remains elusive. In this study, we examined alteration of the ethylene response by the dominant, ethylene-insensitive $etr1\text{-}1^{(C65Y)}$ and $ers1\text{-}1^{(I62P)}/ers1^{C65Y}$ transgenes in receptor-defective mutants to evaluate the functional significance of the receptor cooperativity in ethylene signaling. Our results indicate that the presence of multiple ethylene receptors is essential for the formation of differential receptor cooperation. We hypothesize that the ethylene receptor signal output by the receptor cooperation differentially represses ethylene responses so that a plant can respond to a wide range of ethylene concentrations and exhibits tissue-specific ethylene responsiveness.

RESULTS

etr1-1 Represses Ethylene Responses Primarily Independent of Other Receptor Genes

Etiolated Arabidopsis seedlings exhibit a long hypocotyl and root; ethylene treatment results in inhibition of hypocotyl and root growth and promotes curvature of the apical hook. These growth alterations are termed the "ethylene triple-response phenotype," which is widely used to score for the degree of ethylene response by measurement of hypocotyl length (Guzmán and Ecker, 1990; Bleecker et al., 1998). A previous study indicates that *ETR1* alone actively represses constitutive ethylene responses in air-grown plants whereas *ERS1* alone does not (Liu et al., 2010). In this study, we examined whether the dominant, ethylene-insensitive *etr1-1* may repress ethylene responses in the absence of other receptor genes (Fig. 1A).

ers1-2 is a hypomorph and *ers1-3* a null mutant (Gao et al., 2003; Xie et al., 2006; Qu et al., 2007). The receptor quintuple mutants *etr1 ers1-2 etr2 ein4 ers2* and *etr1 ers1-3 etr2 ein4 ers2*, respectively designated (*ers1-2*) 5LOF and (*ers1-3*)5LOF (where LOF = loss of function), show similar and extreme growth inhibition in air (Liu et al., 2010). Germinated in air, the seedling constitutive triple-response phenotype of (*ers1-2*) 5LOF and (*ers1-3*)5LOF was substantially rescued by the genomic *etr1-1* (designated *getr1-1*) transgene. Ethylene-induced hypocotyl shortening was observed in wild-type (Columbia [Col-0]) seedlings but not in (*ers1-2*)5LOF and (*ers1-3*)5LOF that expressed *getr1-1* (Fig. 1, B and C). Grown under light, ethylene inhibited cotyledon expansion, hypocotyl elongation, and root growth in wild-type (Col-0) seedlings. The ethylene-induced growth inhibition was not observed in (*ers1-2*) 5LOF and (*ers1-3*)5LOF that expressed *getr1-1* (Fig. 1D). At the adult stage, the receptor quintuple mutants developed an extremely small rosette and exhibited an early-senescence phenotype. Expression of *getr1-1*

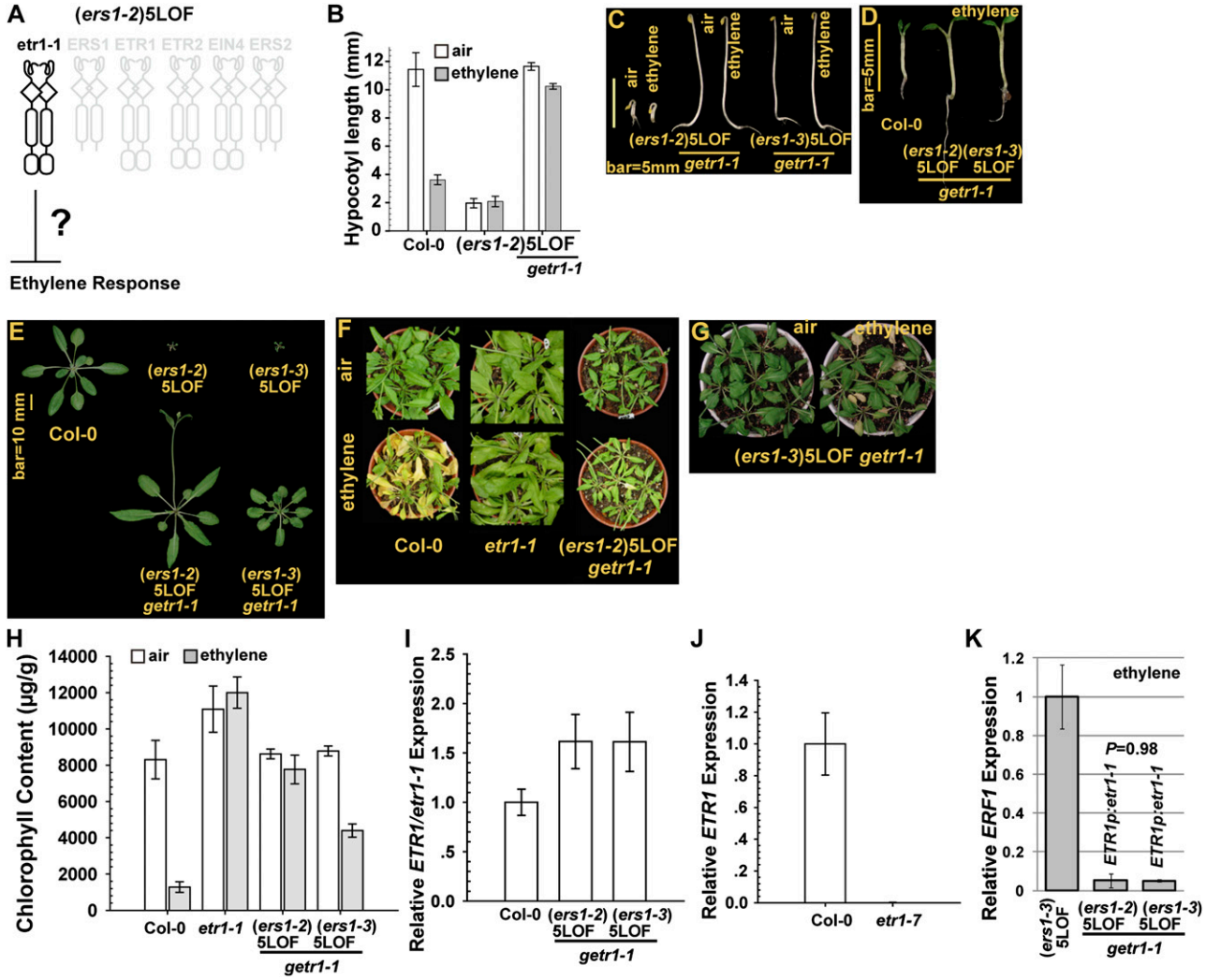

Figure 1. Ethylene response phenotypes of receptor quintuple mutants that express *getr1-1*. A, Diagrammatic illustration of the receptor composition of the quintuple mutant that expressed *getr1-1*. Defective receptors are illustrated in gray. The question mark indicates the ethylene response status. B, Seedling hypocotyl length of the quintuple mutants, with and without the transgene, and the wild type (Col-0). C and D, Phenotypes of seedlings grown in the dark (C) and in the light (D). E, Rosette phenotypes of the wild type (Col-0), receptor quintuple mutants, and the mutants that expressed *getr1-1*. F, Ethylene-induced leaf senescence phenotypes of the wild type (Col-0), *etr1-1*, and (*ers1-2*)5LOF *getr1-1*. G, Leaf senescence phenotype of (*ers1-3*) 5LOF *getr1-1*. H, Leaf chlorophyll content of the wild type (Col-0), *etr1-1*, and quintuple mutants that expressed *getr1-1*. I and J, Relative expression levels of *ETR1* (in Col-0) and *etr1-1* (in quintuple mutants; I) and *ETR1* (in Col-0 and *etr1-7*; J). K, Relative *ERF1* expression level of (*ers1-3*)5LOF and the quintuple mutants that expressed *getr1-1*. Error bars indicate the SD of each measurement ($n \geq 30$ for seedling hypocotyl measurement; $n = 3 \times 3$ for gene expression and chlorophyll measurement).

substantially rescued rosette growth; (*ers1-2*)5LOF *getr1-1* plants displayed early flowering and were larger than (*ers1-3*)5LOF *getr1-1* plants (Fig. 1E). A previous study indicated that the early-flowering phenotype could be a trait inherited from some loci in the Wassilewskija background, which the *ers1* allele comes from (Liu et al., 2010). The larger rosette of (*ers1-2*) 5LOF *getr1-1* plants compared with that of (*ers1-3*)5LOF *getr1-1* plants could be because of leakiness of the *ers1-2* mutation.

Other aspects of the ethylene response were examined in the quintuple mutants that expressed *getr1-1*. Wild-type (Col-0) plants showed a severe leaf senescence phenotype after ethylene treatment compared with the phenotype of the ethylene-insensitive *etr1-1* (Fig. 1F). Expression of *getr1-1* substantially prevented the ethylene-induced leaf senescence phenotype of (*ers1-2*)5LOF, although a number of older leaves showed partial senescence after ethylene treatment (Fig. 1F). (*ers1-3*)5LOF *getr1-1* plants displayed a more promising ethylene-induced leaf senescence phenotype in several older leaves (Fig. 1G). The degree of leaf senescence was quantitatively scored by measurement of the chlorophyll content. Chlorophyll content

was not affected in the *etr1-1* mutant but was substantially reduced (by up to 85%) in wild-type (Col-0) plants. The chlorophyll content of (*ers1-2*)5LOF *getr1-1* leaves was slightly reduced (by 10%) after ethylene treatment, whereas that of (*ers1-3*)5LOF *getr1-1* leaves was reduced to a greater extent (by 50%; Fig. 1H).

Whether the differential changes in the chlorophyll degradation of (*ers1-2*)5LOF *getr1-1* and (*ers1-3*)5LOF *getr1-1* plants were a result of differential expression of the *getr1-1* transgene was investigated. The *etr1-7* mutation is an intragenic suppressor of *etr1-1* and thus has two lesions: one is the *etr1-1* mutation and the other is the W74stop early termination. The primer set for real-time fluorescence quantitative reverse transcription (qRT)-PCR was designed to match the sequence of the wild-type *ETR1* and *etr1-1* but not that of *etr1-7*. Our data showed that *getr1-1* expression was identical in the two transformation mutants (Student's *t* test, $P > 0.05$; Fig. 1I). As a control, *etr1-7* expression was not detected by the same primer set, which indicated the validity of the qRT-PCR analysis (Fig. 1J).

Ethylene Response Factor1 (*ERF1*) is a primary target of the ethylene signal (Chao et al., 1997; Solano and Ecker, 1998). Thus, *ERF1* expression is ideal as a measure of the degree of ethylene responsiveness. Results from the qRT-PCR analysis showed that expression of the ethylene-insensitive *getr1-1* transgene substantially attenuated *ERF1* levels in the ethylene-treated receptor quintuple mutants (Fig. 1K). *ERF1* expression in (*ers1-2*)5LOF *getr1-1* and (*ers1-3*)5LOF *getr1-1* plants was identical (Student's *t* test, $P = 0.98$). These results suggest that *ERF1* level and the severity of the growth inhibition phenotype are tightly correlated.

The results of this study showed that the dominant ethylene-insensitive *etr1-1* alone largely, but not completely, repressed different ethylene responses in all developmental stages. The larger rosette size and lower degree of leaf senescence of (*ers1-2*)5LOF *getr1-1* plants compared with those of (*ers1-3*)5LOF *getr1-1* plants may indicate that the remaining, small amount of ERS1 in (*ers1-2*)5LOF can partially support the dominant mutant receptor etr1-1 functions.

ETR1 Supports Ethylene Insensitivity Conferred by *ers1-1*$^{(I62P)}$

Neither the prevention of endogenous ethylene biosynthesis by L-α-(2-aminoethoxyvinyl)glycine nor competition for ethylene binding by 1-methylcyclopropene (1-MCP) is able to rescue effectively the seedling growth defects of *etr1 etr2 ein4 ers2* [designated (*ERS1*)4LOF; Supplemental Fig. S1; Liu et al., 2010), which implied that repression of the constitutive ethylene response by *ERS1* failed. We next reciprocally tested whether the dominant ethylene-insensitive receptor gene *ers1-1*$^{(I62P)}$ alone is sufficient to repress ethylene responses when other receptor genes are absent.

ers1-1$^{(I62P)}$ is an artificially created dominant mutation that results in the I62P substitution and confers ethylene insensitivity (Hua et al., 1995). The genomic *ers1-1*$^{(I62P)}$ transgene is designated *gers1-1*$^{(I62P)}$. Air-grown *gers1-1*$^{(I62P)}$ (Col-0; lines *L42* and *L51*) seedlings had a much longer hypocotyl than wild-type seedlings, whereas ethylene treatment slightly reduced the seedling hypocotyl length of *gers1-1*$^{(I62P)}$ (Col-0; *L42* and *L51*) seedlings (Student's *t* test, $P < 0.01$). These results indicated that the *gers1-1*$^{(I62P)}$ transgene substantially prevented ethylene-induced hypocotyl shortening in wild-type (Col-0) seedlings (Fig. 2A). Expression of *gers1-1*$^{(I62P)}$ in the (*ETR1*)4LOF quadruple mutant also largely prevented ethylene-induced hypocotyl shortening (*L30* and *L31*), although the seedlings were slightly ethylene responsive based on hypocotyl length (Student's *t* test, $P < 0.01$). (*ETR1/etr1-7*) *ers1-2 etr2 ein4 ers2*, a quintuple mutant heterozygous for *ETR1*, is designated (*ETR1/-*)4LOF. As expected, the expression of *gers1-1*$^{(I62P)}$ in (*ETR1/-*)4LOF plants also prevented ethylene-induced seedling hypocotyl shortening [line *L1*, designated (*ETR1/-*)4LOF *gers1-1*$^{(I62P)}$ *L1*]. *etr1 ers1-2 etr2 ein4 ers2 gers1-1*$^{(I62P)}$ is a sibling of (*ETR1/-*)4LOF *gers1-1*$^{(I62P)}$ *L1*, designated (*ers1-2*)5LOF *gers1-1*$^{(I62P)}$ *L1*, that does not carry any wild-type receptor gene and exhibited seedling hypocotyl shortening regardless of ethylene treatment (Fig. 2A). The strong growth inhibition of air-grown (*ers1-2*)5LOF and (*ers1-2*)5LOF *gers1-1*$^{(I62P)}$ *L1* seedlings indicated strong constitutive ethylene responses of the two mutants so that exogenous ethylene treatment no longer exerted any inhibition of seedling growth. These results indicated that the dominant ethylene-insensitive mutant receptor ers1-1$^{(I62P)}$ failed to repress constitutive ethylene responses or to confer ethylene insensitivity in the absence of other wild-type ethylene receptors.

Consistent with these results, light-grown seedlings of (*ETR1/-*)4LOF *gers1-1*$^{(I62P)}$ *L1* showed expanded cotyledons, and primary root and hypocotyl growth appeared to be normal. Its sibling, (*ers1-2*)5LOF *gers1-1*$^{(I62P)}$ *L1*, phenotypically resembled (*ERS1*)4LOF and (*ers1-2*)5LOF plants and exhibited strong growth inhibition (Fig. 2B). At the adult stage, (*ers1-2*)5LOF plants displayed an early leaf senescence phenotype and produced an extremely small rosette. As expected, (*ETR1/-*)4LOF *gers1-1*$^{(I62P)}$ *L1* and its (*ETR1*)4LOF *gers1-1*$^{(I62P)}$ *L1* sibling showed normal and similar rosette growth, whereas (*ers1-2*)5LOF *gers1-1*$^{(I62P)}$ *L1* exhibited strong growth inhibition but with a slightly larger rosette than the untransformed (*ers1-2*)5LOF plants (Fig. 2C). Ethylene treatment resulted in a severe leaf senescence phenotype in (*ers1-2*)5LOF *gers1-1*$^{(I62P)}$ *L1*; in contrast, the senescence phenotype of (*ETR1/-*)4LOF *gers1-1*$^{(I62P)}$ *L1* was weak (Fig. 2D). We next quantified the degree of leaf senescence by measurement of chlorophyll content (Fig. 2E). Chlorophyll content of the *ers1-1* mutant was unaltered (Student's *t* test, $P = 0.53$), slightly reduced (by 30%) in (*ETR1/-*)4LOF *gers1-1*$^{(I62P)}$ *L1* plants, and substantially reduced (by about 60%) in (*ers1-2*)5LOF *gers1-1*$^{(I62P)}$ *L1* plants after ethylene treatment. Notably, the chlorophyll content of air-grown, untreated (*ers1-2*)5LOF *gers1-1*$^{(I62P)}$

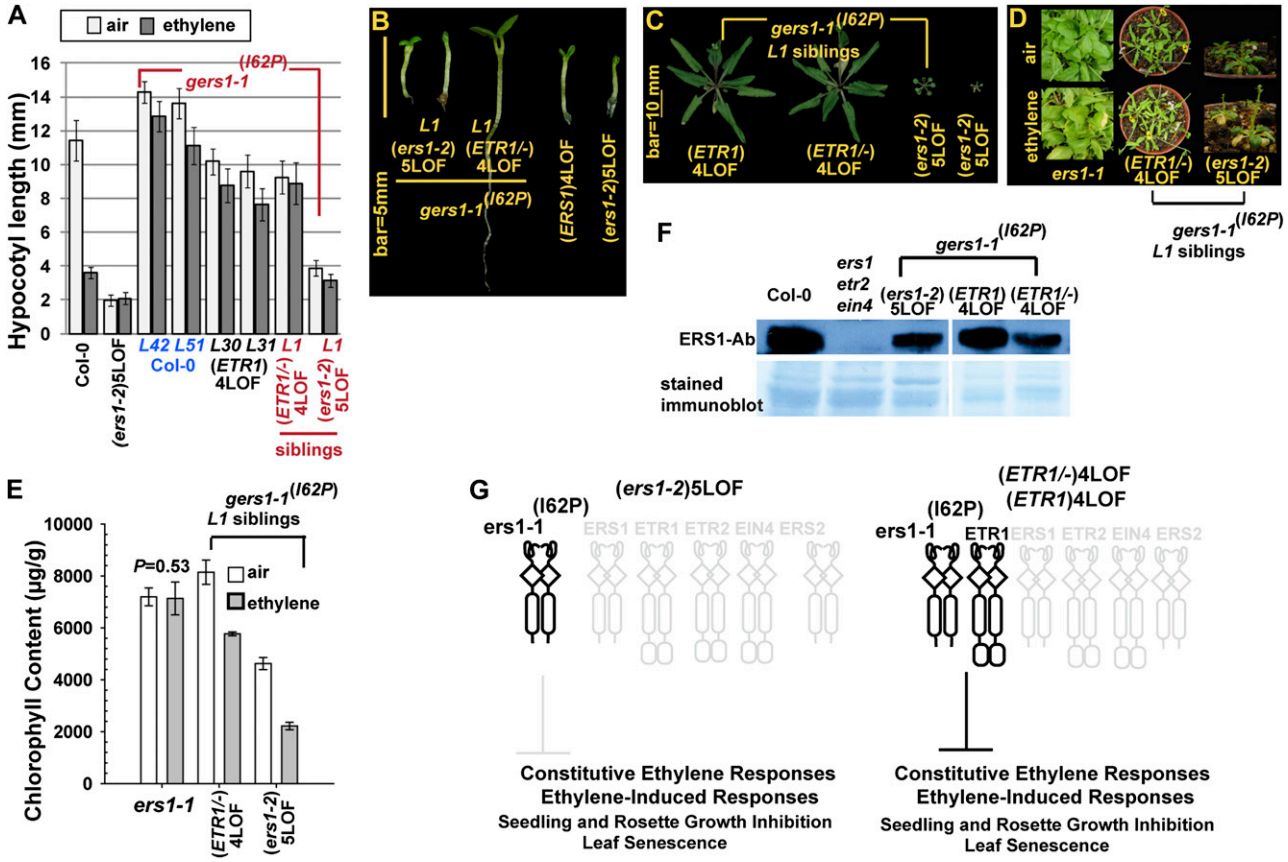

Figure 2. Ethylene responses of receptor mutants that expressed *gers1-1*$^{(I62P)}$. A, Seedling hypocotyl length for transformation lines carrying *gers1-1*$^{(I62P)}$. *L42* and *L51* (in blue) are wild-type (Col-0) *gers1-1*$^{(I62P)}$ transformation lines. *L30* and *L31* (in black) are (*ETR1*)4LOF *gers1-1*$^{(I62P)}$ transformation lines. (*ETR1*/-)4LOF *gers1-1*$^{(I62P)}$ *L1* and (*ers1-2*)5LOF *gers1-1*$^{(I62P)}$ *L1* (in red) are siblings. B and C, Seedling (B) and adult (C) phenotypes of receptor mutants and the mutants carrying *gers1-1*$^{(I62P)}$, where (*ETR1*)4LOF *gers1-1*$^{(I62P)}$ *L1* is a sibling of (*ETR1*/-)4LOF *gers1-1*$^{(I62P)}$ *L1*. D, Leaf senescence phenotype for receptor mutants carrying *gers1-1*$^{(I62P)}$. E, Chlorophyll measurement for *ers1-1* and transformation mutants carrying *gers1-1*$^{(I62P)}$ before (air) and after (ethylene) ethylene treatment. F, Immunoassay for ers1-1$^{(I62P)}$ protein expression. ERS1-Ab, Immunosignal by ERS1 antibodies; stained immunoblot, a membrane subjected to immunoassay was stained with Coomassie blue to show relative protein amounts. The immunoassay was performed with the same blot. G, Diagrammatic illustration of the receptor composition and ethylene response status of the transformation mutants that expressed *gers1-1*$^{(I62P)}$. The ETR1-ERS1 association, probably via the GAF domain (Gao et al., 2003), may facilitate the ers1-1$^{(I62P)}$ signal output. Error bars indicate the SD for each measurement ($n \geq 30$ for hypocotyl measurement; $n = 3 \times 3$ for chlorophyll measurement).

L1 plants was already much lower than that of wild-type plants (by 34%) and (*ETR1*/-)4LOF *gers1-1*$^{(I62P)}$ *L1* plants (by 44%), which implied that the constitutive ethylene response of (*ers1-2*)5LOF *gers1-1*$^{(I62P)}$ *L1* was elevated and sufficient to promote chlorophyll degradation.

Given that the *gers1-1*$^{(I62P)}$ transgene was unable to rescue the growth defects of the receptor quintuple mutant, we examined whether the ers1-1$^{(I62P)}$ protein was expressed. Figure 2F shows the immunoassay performed with the same blot. *ers1-2 etr2 ein4* is an *ERS1*-defective mutant in which ERS1 was not immunologically detectable. In contrast, ERS1 and ers1-1$^{(I62P)}$ proteins were immunologically detectable in wild-type (Col-0) plants and *gers1-1*$^{(I62P)}$ transformation lines that lack *ERS1*, respectively (Fig. 2F).

The receptor composition and differential repression of ethylene responses by *gers1-1*$^{(I62P)}$ in this study are diagrammatically summarized in Figure 2G. Our data indicated that the dominant mutant receptor ers1-1$^{(I62P)}$ alone was insufficient to repress the constitutive ethylene response in air and to confer ethylene insensitivity. ETR1 alone was able to support ers1-1$^{(I62P)}$ functions to a large extent. ETR1 and ERS1 can form a heterodimer (Gao et al., 2008); conceivably, the ETR1-ERS1 association may facilitate the ERS1 receptor signal output.

Receptor Mutant Growth Is Differentially Rescued by *gers1-1*$^{(I62P)}$ in Combination with a Wild-Type Receptor Gene

Our data indicated that ethylene insensitivity conferred by the dominant ethylene-insensitive gene *ers1-1*$^{(I62P)}$ depended at least on *ETR1*. We next evaluated whether other wild-type receptors also support

the repression of ethylene responses by the dominant mutant receptor ers1-1$^{(I62P)}$.

etr1 ers1-2 exhibits strong growth inhibition at all developmental stages and an extremely small rosette size (Wang et al., 2003). Expression of *gers1-1*$^{(I62P)}$ substantially rescued the extreme growth defects of *etr1 ers1-2* (transformation lines *L10* and *L30* in Fig. 3A), which implied that *gers1-1*$^{(I62P)}$ repressed constitutive ethylene responses in combination with the remaining ethylene receptor genes.

To evaluate the ability of each wild-type receptor gene to support ethylene insensitivity conferred by the dominant *gers1-1*$^{(I62P)}$, we expressed the *gers1-1*$^{(I62P)}$ transgene in each of the receptor quadruple mutants. The quadruple receptor mutants *etr1 etr2 ein4 ers2*, *etr1 ers1-2 ein4 ers2*, *etr1 ers1-2 etr2 ers2*, and *etr1 ers1-2 etr2 ein4* were designated (*ERS1*)4LOF, (*ETR2*)4LOF, (*EIN4*)4LOF, and (*ERS2*)4LOF, respectively. *gers1-1* $^{(I62P)}$ was introduced from the common transgene donor (*ETR1*/-)4LOF *gers1-1*$^{(I62P)}$ *L1* to each of the quadruple mutants by genetic crosses. Thus, the *gers1-1*$^{(I62P)}$ transgene was expressed at the same locus in each quadruple mutant, as depicted in Figure 3B.

(*ETR1*)4LOF displays mild growth defects at all developmental stages (Liu et al., 2010). Air-grown (*ERS1*)4LOF, (*ETR2*)4LOF, (*EIN4*)4LOF, and (*ERS2*) 4LOF plants showed the typical seedling triple-response phenotype, and each genotype displayed a short seedling hypocotyl and primary root (Fig. 3C). Among these five quadruple mutants, expression of *gers1-1*$^{(I62P)}$ had the least effect on rescuing the growth of etiolated (*ERS1*)4LOF seedlings. In contrast, *gers1-1*$^{(I62P)}$ rescued the growth defects of etiolated (*ETR1*)4LOF, (*ETR2*)4LOF, (*EIN4*)4LOF, and (*ERS2*) 4LOF seedlings to different extents, and ethylene treatment had little effect on hypocotyl elongation in these mutants that expressed the transgene. Among the quadruple mutants that carried the *gers1-1*$^{(I62P)}$ transgene, (*ETR1*)4LOF *gers1-1*$^{(I62P)}$ and (*EIN4*)4LOF *gers1-1*$^{(I62P)}$ seedlings displayed the longest hypocotyls regardless of ethylene treatment (Fig. 3, C and D). Except for (*ETR1*)4LOF, seedlings of light-grown receptor quadruple mutants displayed a strong growth inhibition phenotype (Fig. 3E). The growth inhibition of these quadruple mutants was rescued to different extents by the *gers1-1*$^{(I62P)}$ transgene (Fig. 3F). Among the five transformation mutants, (*ERS1*)4LOF exhibited the least growth rescue by *gers1-1*$^{(I62P)}$. The growth rescue of these quadruple mutants by *gers1-1*$^{(I62P)}$ was barely affected by ethylene treatment (Fig. 3G).

Consistent with these results, at the adult stage, except for (*ETR1*)4LOF (Liu et al., 2010), these quadruple mutants exhibited severe rosette growth inhibition and an extremely small rosette size. Expression of *gers1-1*$^{(I62P)}$ slightly rescued the growth defects of (*ERS1*)4LOF. As expected, growth rescue of (*ETR2*) 4LOF by *gers1-1*$^{(I62P)}$ was much greater than that of (*ERS1*)4LOF but weaker than that of (*ETR1*)4LOF, (*EIN4*)4LOF, and (*ERS2*)4LOF (Fig. 3H). The differential growth rescue of the five quadruple mutants by *gers1-1*$^{(I62P)}$ may indicate that differential repression of constitutive ethylene responses occurred in the respective mutants.

A relationship between the expression of each receptor gene and corresponding receptor protein amount has been indicated (O'Malley et al., 2005; Gao et al., 2008). We measured the expression level of receptor genes to evaluate whether the differential growth rescue by *gers1-1*$^{(I62P)}$ was a result of an alteration in the amount of the corresponding receptor protein. Receptor gene expression in wild-type (Col-0) plants was standardized to a value of 1. Among the transformation mutants, the expression of *ETR1* and *ETR2* was marginally reduced (Student's *t* test, $P < 0.001$) and that of *EIN4* was unchanged (Student's *t*-test, $P = 0.46$; Fig. 3I). In (*ERS1*)4LOF *gers1-1*$^{(I62P)}$ plants, expression of *ERS1* and *ers1-1*$^{(I62P)}$ was elevated by about 6-fold, whereas the expression level of *ERS2* in (*ERS2*)4LOF *gers1-1*$^{(I62P)}$ was strongly reduced.

These results showed that each of the wild-type receptor genes differentially supported ethylene insensitivity conferred by the dominant *gers1-1*$^{(I62P)}$. In combination with *ETR1* or *EIN4*, *ers1-1*$^{(I62P)}$ had the greatest effect, whereas *ERS1* showed the weakest effect, on the growth rescue and prevention of ethylene-induced growth inhibition. Strongly reduced expression of *ERS2* was still sufficient to support *gers1-1*$^{(I62P)}$ functions, whereas an elevated *ERS1* expression level was not. The amount of EIN4 is indicated to be the lowest among the five receptor members (Gao et al., 2008), whereas *EIN4* supported *gers1-1*$^{(I62P)}$ functions to a large extent. Our data indicated that the receptor identity, rather than the receptor amount, had important roles in ers1-1$^{(I62P)}$ functions. The growth defects of the quadruple mutants differentially rescued by *gers1-1*$^{(I62P)}$ may indicate that the dominant mutant receptor ers1-1$^{(I62P)}$ forms differential cooperation with the other wild-type receptors.

Combinatorial Effects of *gers1-1*$^{(I62P)}$ and Other Wild-Type Receptor Genes on Ethylene Responses

Our results here show that the dominant mutant receptor gene *ers1-1*$^{(I62P)}$ has differential effects on growth of the receptor mutants when combined with a wild-type receptor gene. We next examined whether the alterations in growth phenotype correlate with the degree of ethylene response.

Results from the qRT-PCR analysis showed that after ethylene treatment, *ERF1* levels in (*ETR1*)4LOF, (*EIN4*) 4LOF, and (*ERS2*)4LOF transformation mutants that expressed *gers1-1*$^{(I62P)}$ were substantially lower than that in wild-type (Col-0) plants (Fig. 4A). The expression of *ERF1* in (*ETR2*)4LOF *gers1-1*$^{(I62P)}$ was slightly higher than that in wild-type plants. As expected, (*ERS1*) 4LOF *gers1-1*$^{(I62P)}$ showed the highest ethylene-induced *ERF1* expression. These results showed that the degree of growth inhibition and *ERF1* expression level were strongly correlated in the transformation mutants.

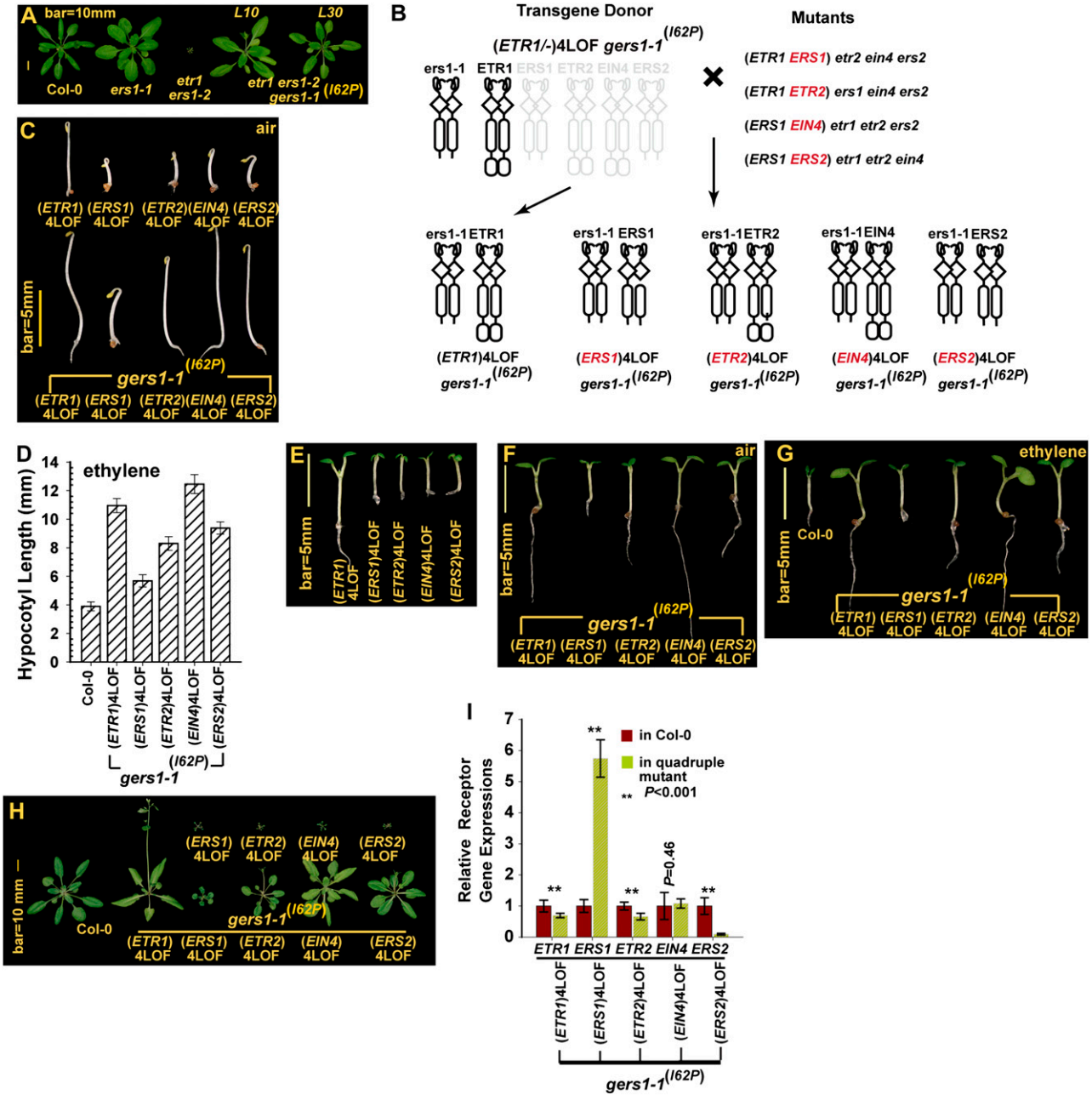

Figure 3. Mutant phenotypes of *etr1 ers1* and receptor quadruple mutants that expressed *gers1-1*$^{(I62P)}$. A, Rosette phenotype of *etr1 ers1* and the mutant that expressed *gers1-1*$^{(I62P)}$. B, Diagrammatic illustration showing the genetic crosses by which the quadruple mutants that carry the *gers1-1*$^{(I62P)}$ transgene were obtained. The receptor gene retained after the cross is indicated in red. C, Etiolated seedling phenotypes of air-grown quadruple mutants and corresponding mutants carrying *gers1-1*$^{(I62P)}$. D, Hypocotyl measurement of ethylene-treated transformation mutants. E, Seedling phenotypes of light-grown quadruple mutants. F and G, Seedling phenotypes of corresponding transformation lines carrying *gers1-1*$^{(I62P)}$ in air (F) and ethylene (G). H, Rosette phenotypes of the quadruple mutants and corresponding transformation mutants. I, Relative expression of the remaining wild-type receptor genes in each quadruple mutant that expressed *gers1-1*$^{(I62P)}$. Error bars indicate the SD for the hypocotyl length ($n \geq 30$) or gene expression measurement ($n = 3 \times 3$). ** $P < 0.001$ compared with the wild type (Col-0) by Student's *t* test. For the seedling growth analyses, 10 μL L^{-1} ethylene was applied.

The severity of the ethylene-induced leaf senescence phenotype and the reduction of leaf chlorophyll content in (*ETR1*/-)4LOF *gers1-1*$^{(I62P)}$ were weak (Fig. 2). We next examined the alteration in leaf senescence phenotype of ethylene-treated (*ERS1*)4LOF, (*ETR2*)4LOF, (*EIN4*)4LOF, and (*ERS2*)4LOF that expressed *gers1-1*$^{(I62P)}$. After ethylene treatment for 4 d, all of the transformation mutants exhibited a leaf senescence

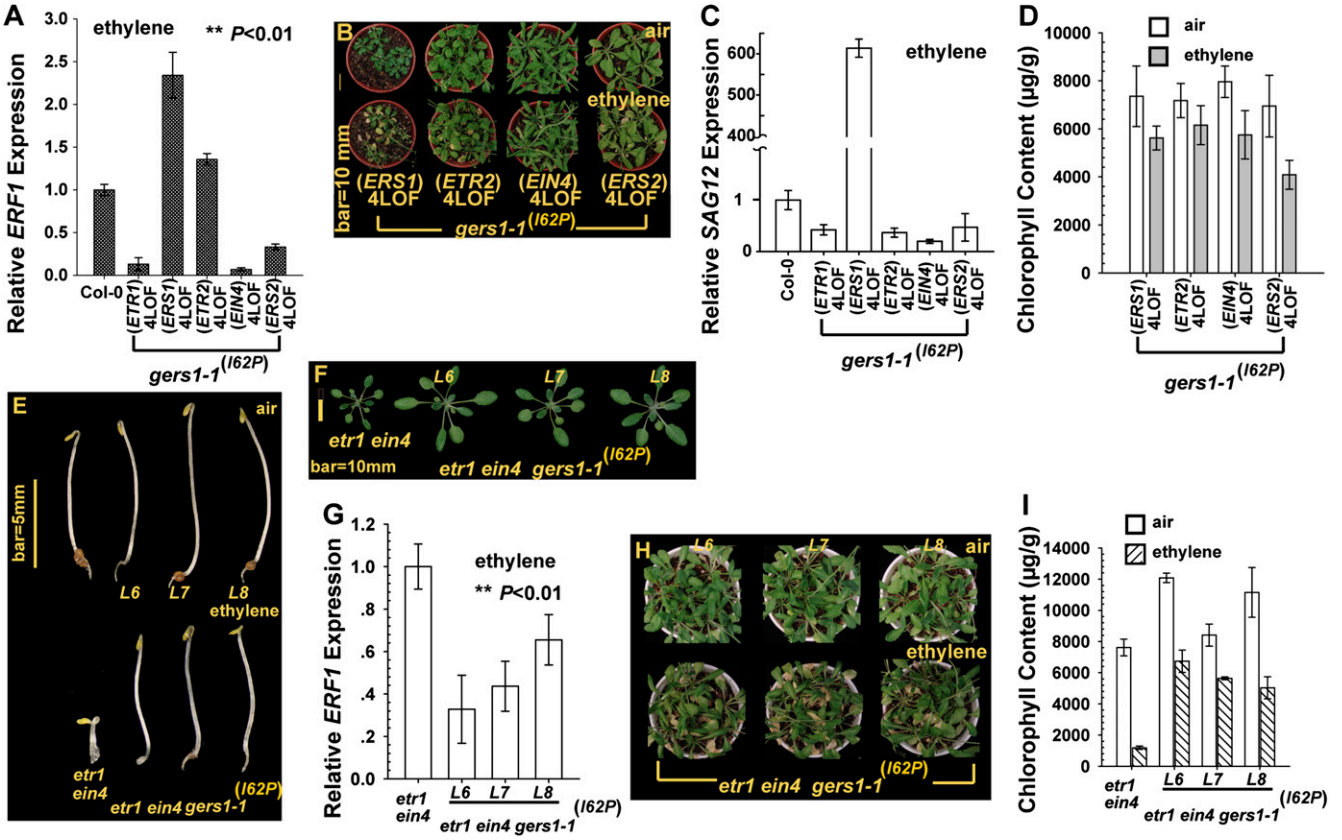

Figure 4. Evaluation of the ethylene response affected by the expression of *gers1-1*$^{(I62P)}$ in receptor quadruple mutants and *etr1 ein4*. A to D, *ERF1* expression (A), leaf senescence phenotype (B), measurement of *SAG12* expression (C), and leaf chlorophyll content (D) of individual receptor quadruple mutants that expressed *gers1-1*$^{(I62P)}$. E to I, Seedling triple-response phenotype (E), rosette growth (F), *ERF1* expression (G), leaf senescence phenotype (H), and leaf chlorophyll content (I) of *etr1 ein4* and *etr1 ein4 gers1-1*$^{(I62P)}$ transformation lines (*L6*, *L7*, and *L8*). Error bars indicate the SD for each measurement ($n = 3 \times 3$ for gene expression or chlorophyll measurement). ** $P < 0.01$ by Student's *t* test for each *ERF1* measurement compared with the wild type (Col-0). For the seedling phenotype analyses, air and ethylene indicate the absence and presence of ethylene (10 μL L^{-1}) treatment, respectively. For the leaf senescence test and chlorophyll measurement, air and ethylene indicate before and after ethylene treatment (100 μL L^{-1}).

phenotype in older leaves (Fig. 4B). *Senescence-Associated Gene12* (*SAG12*) is specifically induced during the progression of leaf senescence (Grbić and Bleecker, 1995; Grbić, 2003). The qRT-PCR analysis was carried out 36 h after the completion of ethylene treatment for measurement of the *SAG12* expression level. In comparison with the expression level in wild-type (Col-0) plants, *SAG12* was highly induced in (*ERS1*)4LOF *gers1-1*$^{(I62P)}$ plants but was attenuated to different extents in the other quadruple mutants that expressed *gers1-1*$^{(I62P)}$. The *SAG12* expression level of (*ETR1*)4LOF *gers1-1*$^{(I62P)}$, (*ETR2*)4LOF *gers1-1*$^{(I62P)}$, and (*ERS2*)4LOF *gers1-1*$^{(I62P)}$ transformation mutants was identical (*F* test, $P = 0.38$) and marginally higher than that of (*EIN4*)4LOF *gers1-1*$^{(I62P)}$ plants (*F* test, $P = 0.016$; Fig. 4C). To quantify the degree of leaf senescence, the leaf chlorophyll content was measured at the completion of ethylene treatment for 4 d. Each of the transformation mutants showed a minor reduction in leaf chlorophyll content (Fig. 4D). This minor reduction was also observed in (*ERS1*)4LOF *gers1-1*$^{(I62P)}$, which displayed the highest *SAG12* and *ERF1* expression levels. Nevertheless, this discrepancy is not inconsistent with the argument that ethylene can differentially induce many, but not all, aspects of the ethylene response in (*ERS1*)4LOF *gers1-1*$^{(I62P)}$.

Our results lend support to the argument that ethylene responses can be differentially repressed by the dominant *ers1-1*$^{(I62P)}$ in combination with a wild-type receptor gene. The relatively higher *ERF1* expression and low *SAG12* expression and chlorophyll degradation of (*ETR2*)4LOF *gers1-1*$^{(I62P)}$ in response to ethylene treatment may indicate that the combination of *ers1-1*$^{(I62P)}$ and *ETR2* has differential effects on ethylene responses. Up-regulation of *SAG12* and delayed chlorophyll degradation of (*ERS1*)4LOF *gers1-1*$^{(I62P)}$ plants may indicate a delay in the progression of leaf senescence in that transformation mutant but not during early stages of leaf senescence. Thus, ers1-1$^{(I62P)}$ may cooperate with an ethylene receptor to specifically regulate certain aspects of the ethylene response; this argument is in line with the

implication that ERS1 may participate in unique downstream responses (Chen et al., 2010).

The results showed that *ers1-1*$^{(I62P)}$ was sufficient to repress various ethylene responses when coupled with *ETR1* or *EIN4*; therefore, we examined whether the absence of both *ETR1* and *EIN4* may attenuate *ers1-1*$^{(I62P)}$ functions. *gers1-1*$^{(I62P)}$ was introduced to *etr1 ein4-7* by transformation, and three independent lines (*L6*, *L7*, and *L8*) were characterized. Notably, the *ein4-7* mutant that was used in this study exhibits ethylene hypersensitivity (Hua and Meyerowitz, 1998). Etiolated, air-grown *etr1 ein4* seedlings showed hypocotyl growth inhibition, and expression of *gers1-1*$^{(I62P)}$ slightly rescued the growth inhibition. Ethylene treatment caused substantial hypocotyl shortening of *etr1 ein4* seedlings but had a minor effect on *etr1 ein4 gers1-1*$^{(I62P)}$ lines (Fig. 4E). At the adult stage, the growth defects of *etr1 ein4* were moderately rescued by the *gers1-1*$^{(I62P)}$ transgene (Fig. 4F).

The degree of ethylene response of *etr1 ein4* and the transformation lines was scored by measurement of the *ERF1* expression level induced by ethylene. Consistent with the growth-defect phenotype, ethylene-induced *ERF1* expression was moderately attenuated in *etr1 ein4 gers1-1*$^{(I62P)}$ lines by 30% to 60% (Fig. 4G). The partial growth rescue of *etr1 ein4* by *gers1-1*$^{(I62P)}$ was possibly supported by the remaining receptor genes. Leaf senescence was assessed as a measure of the degree of ethylene response in the *etr1 ein4* and *etr1 ein4 gers1-1*$^{(I62P)}$ lines. Upon completion of ethylene treatment for 4 d, *etr1 ein4 gers1-1*$^{(I62P)}$ lines exhibited a weaker leaf senescence phenotype and showed a higher leaf chlorophyll content than those of *etr1 ein4*. The chlorophyll content of *etr1 ein4* decreased to 15.5% and that of *etr1 ein4 gers1-1*$^{(I62P)}$ decreased to 45% to 67% relative to that before ethylene treatment (Fig. 4, H and I). The *etr1 ein4* leaf senescence phenotype is shown in Figure 5H.

These results showed that the dominant *ers1-1*$^{(I62P)}$ ethylene receptor gene was able to partly confer ethylene insensitivity in the absence of both *ETR1* and *EIN4*, which was consistent with results for the transformation mutants that indicated that other receptor genes differentially support *ers1-1*$^{(I62P)}$ functions. Both *etr1* and *ein4-7* single loss-of-function mutants exhibit hypersensitivity to ethylene (Hua and Meyerowitz, 1998); therefore, we do not exclude the possibility that ETR1 and EIN4 may have unique roles in ers1-1$^{(I62P)}$ functions.

The Repression of Ethylene Responses by *gers1*^{C65Y} Is Primarily Dependent on *ETR1* and *EIN4*

Expression of the dominant *etr1-1* and *ers1-1*$^{(I62P)}$ receptor genes differentially repressed the ethylene response in combination with other wild-type receptor genes. Notably, the *etr1-1* mutation results in the C65Y substitution and *ers1-1*$^{(I62P)}$ results in the I62P substitution, and these might behave differently. We introduced the C65Y substitution into ERS1 and examined whether the resulting mutant receptor ers1^{C65Y} can confer ethylene insensitivity in wild-type and *etr1 ein4* plants. The *gers1*^{C65Y} transgene in Col-0 (*L1*, *L3*, and *L5*) was introduced from *etr1 ein4 gers1*^{C65Y} by genetic crosses so that the transgene was expressed at the same locus in each transformation line.

The *gers1*^{C65Y} mutation was dominant (data not shown) and conferred ethylene insensitivity; ethylene treatment slightly affected the seedling hypocotyl elongation of wild-type (Col-0) transformation lines (*L1* and *L3* but not *L5*) that expressed *gers1*^{C65Y} (Fig. 5, A and B; Student's *t* test, $P < 0.01$). In contrast, when *gers1*^{C65Y} was expressed in *etr1 ein4*, the transgene failed to rescue *etr1 ein4* growth in air and did not prevent ethylene-induced hypocotyl shortening (Fig. 5, C and D). Consistent with this result, light-grown *etr1 ein4 gers1*^{C65Y} seedlings exhibited strong growth inhibition in response to ethylene treatment; the cotyledons were small and compact, and the hypocotyl and root were short (Fig. 5E). At the adult stage, expression of *gers1*^{C65Y} did not rescue the *etr1 ein4* growth defects, and the transformation lines exhibited a small rosette (Fig. 5F).

The effect of *gers1*^{C65Y} on the ethylene response of *etr1 ein4* was evaluated by the measurement of *ERF1* expression levels and scoring the leaf senescence phenotype. After ethylene treatment, *ERF1* expression of *etr1 ein4 gers1*^{C65Y} was similar to that of *etr1 ein4* (Fig. 5G). The *etr1 ein4 gers1*^{C65Y} lines displayed a severe senescence phenotype similar to that of *etr1 ein4* after ethylene treatment (Fig. 5H). Following ethylene treatment, the leaf chlorophyll content of *etr1 ein4* and *etr1 ein4 gers1*^{C65Y} substantially decreased. The chlorophyll content (relative to that before ethylene treatment) of *etr1 ein4* was about 15.5% and that of *etr1 ein4 gers1*^{C65Y} lines ranged from 8% to 28% after ethylene treatment (Fig. 5I).

These results indicated that both *ETR1* and *EIN4* are predominantly required for the repression of ethylene responses by *gers1*^{C65Y}. *ETR1* and *EIN4* may thus have synergistic or additive effects on *gers1*^{C65Y} functions; the other receptor genes appear unable to support *gers1*^{C65Y} functions.

Effects of the *etr1* and *ein4* Loss-of-Function Mutations on Ethylene Insensitivity Conferred by *gers1*^{C65Y}

To evaluate whether *ETR1* and *EIN4* may additively or synergistically act with *gers1*^{C65Y}, we isolated three independent *etr1 gers1*^{C65Y} and *ein4 gers1*^{C65Y} lines from the F3 or higher generations derived from genetic crosses between *etr1 ein4 gers1*^{C65Y} lines and wild-type (Col-0) plants. Transformation lines labeled with the same line number carry the same transgene from a common donor; thus, the *gers1*^{C65Y} transgene is expressed at the same locus.

Both *etr1* and *ein4-7* mutations result in ethylene hypersensitivity, according to the ethylene dose-response assay (Hua and Meyerowitz, 1998; Cancel and Larsen, 2002). Etiolated *etr1 gers1*^{C65Y} seedlings

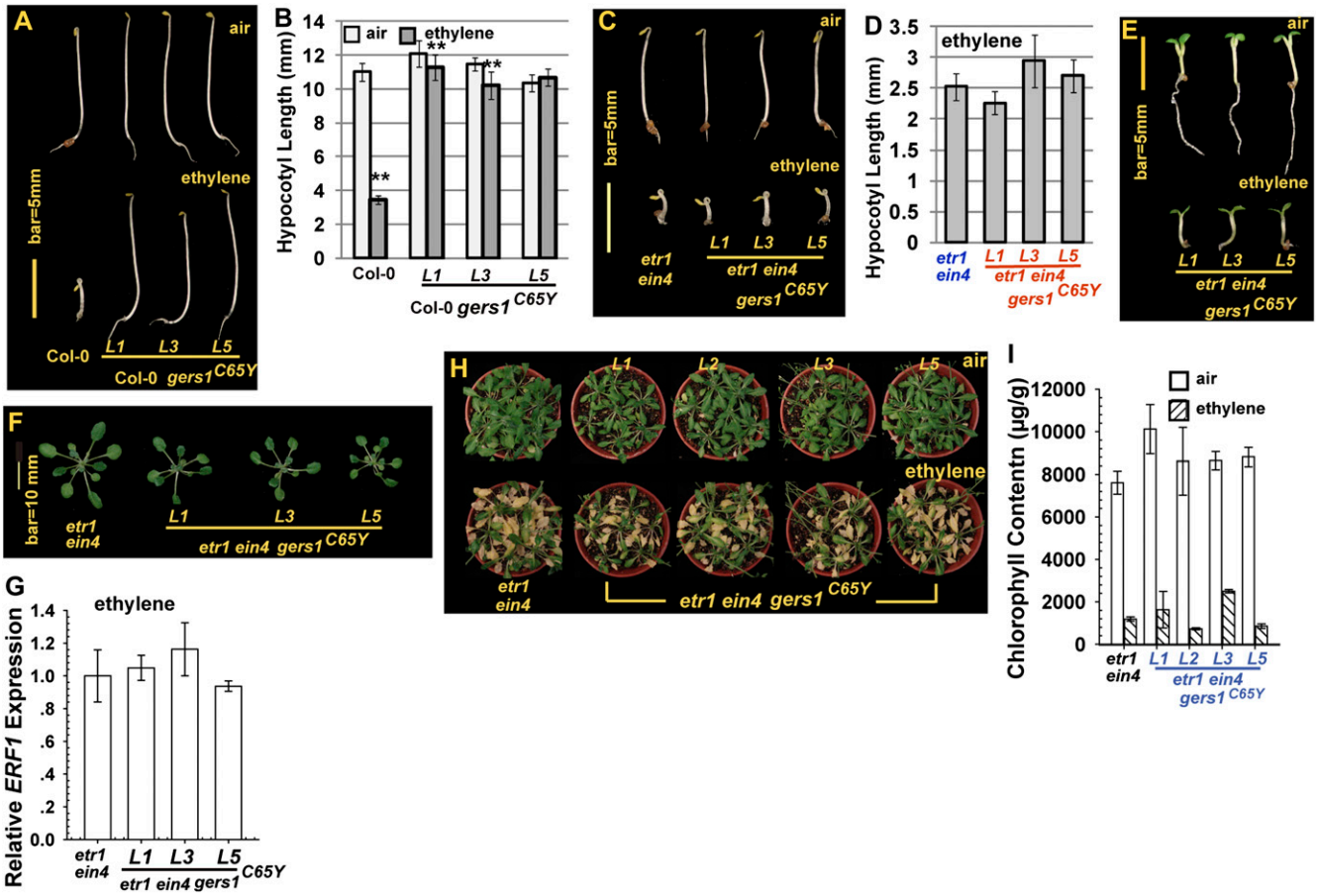

Figure 5. Effects of *etr1 ein4* mutation on the repression of ethylene responses by *gers1*^{C65Y}. A and B, Seedling triple-response phenotype (A) and hypocotyl length (B) of wild-type (Col-0) lines (*L1*, *L3*, and *L5*) that expressed *gers1*^{C65Y}. C to F, Seedling triple-response phenotype (C), hypocotyl length (D), light-grown seedling phenotype (E), and rosette growth (F) of *etr1 ein4* and *etr1 ein4 gers1*^{C65Y} lines. G to I, *ERF1* expression (G), leaf senescence phenotype (H), and leaf chlorophyll content (I) of *etr1 ein4* and *etr1 ein4 gers1*^{C65Y} lines. For the seedling phenotype analyses, air and ethylene indicate the absence and presence of ethylene (10 μL L^{-1}) treatment, respectively. For the leaf senescence test and chlorophyll measurement, air and ethylene indicate before and after ethylene treatment (100 μL L^{-1}). Error bars indicate the SD for each measurement ($n \geq 30$ for the seedling hypocotyl measurement; $n = 3 \times 3$ for gene expression or chlorophyll measurement).

exhibited a long hypocotyl in air, and ethylene treatment did not result in hypocotyl shortening. Air-grown *ein4 gers1*^{C65Y} seedlings (in which *ein4* denoted the *ein4-7* allele) had a long hypocotyl, whereas ethylene treatment resulted in different degrees of hypocotyl shortening (Fig. 6, A and B). Consistent with the seedling phenotype, ethylene barely affected the seedling hypocotyl elongation of *etr1 gers1*^{C65Y}, whereas the hypocotyl length of *ein4 gers1*^{C65Y} seedlings was shortened by 20% to 40% (Fig. 6C). As expected, light-grown *etr1 gers1*^{C65Y} and *ein4 gers1*^{C65Y} seedlings did not show prominent growth defects. Ethylene treatment resulted in partial growth inhibition of *ein4 gers1*^{C65Y} plants, and the cotyledons, hypocotyls, and roots were moderately reduced in size. The effects of ethylene on *etr1 gers1*^{C65Y} growth were minor (Fig. 6D). Results from the qRT-PCR analysis showed that the *ERF1* expression levels of ethylene-treated *etr1 gers1*^{C65Y} and *ein4 gers1*^{C65Y} plants were 5% to 10% and about 17%, respectively, relative to that of ethylene-treated wild-type seedlings (Fig. 6E).

These results show that the lack of *EIN4* moderately weakened ethylene insensitivity conferred by *gers1*^{C65Y}, whereas the lack of *ETR1* did not affect ethylene insensitivity. *etr1 ein4 gers1*^{C65Y} plants displayed strong growth inhibition and enhanced leaf senescence in response to ethylene treatment; therefore, *ETR1* and *EIN4* may have synergistic effects on the ethylene insensitivity conferred by *gers1*^{C65Y}. The *etr1* loss-of-function mutant exhibits stronger ethylene hypersensitivity than *ein4-7* (Hua and Meyerowitz, 1998). Thus, it is more likely that EIN4 identity, rather than EIN4 amount or *ein4-7* ethylene hypersensitivity, affects ers1^{C65Y} functions.

DISCUSSION

Genetic studies indicate that ethylene receptors may activate CTR1 (a mitogen-activated protein kinase

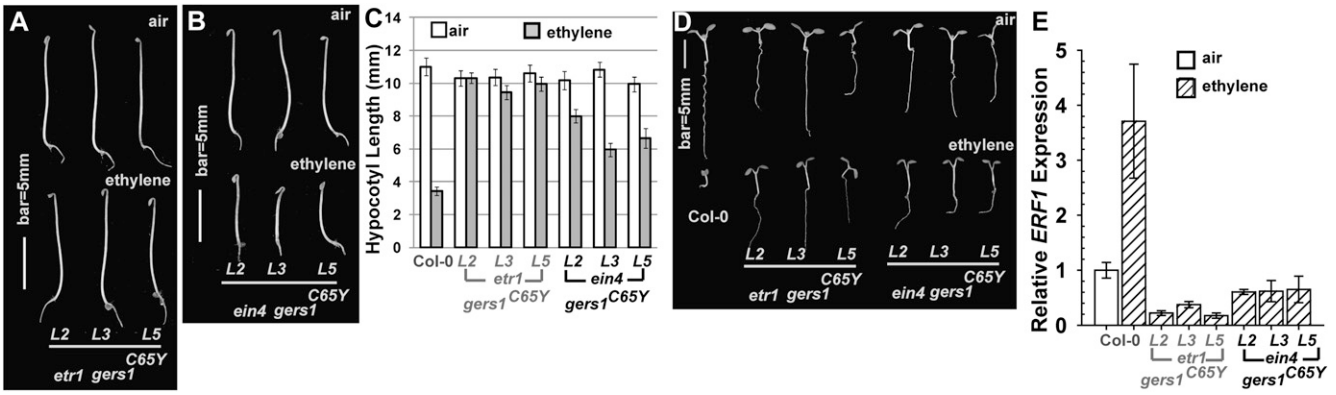

Figure 6. Effects of *etr1* and *ein4* mutations on the inhibition of ethylene responses by *gers1*^{C65Y}. A to C, Etiolated seedling phenotype of *etr1 gers1*^{C65Y} (A) and *ein4 gers1*^{C65Y} (B) lines in air and ethylene and their respective seedling hypocotyl lengths (C). D, Light-grown seedling phenotypes of *etr1* and *ein4* transformation lines carrying *gers1*^{C65Y} in air and ethylene. E, Relative *ERF1* levels measured in *etr1* and *ein4* loss-of-function mutants carrying *gers1*^{C65Y} grown in ethylene. Error bars indicate the SD for each measurement ($n \geq 30$ for the seedling hypocotyl measurement; $n = 3 \times 3$ for gene expression).

kinase kinase) through a mechanism requiring physical association to repress the ethylene response (Hua and Meyerowitz, 1998; Klee, 2004; Wang et al., 2006). Although the five ethylene receptor genes are genetically redundant, the functions of an ethylene receptor gene cannot be compensated by the expression of another, which implies that each receptor gene has unique roles in ethylene signaling (Wang et al., 2003; Klee, 2004; O'Malley et al., 2005; Liu et al., 2010). The ethylene receptors may dimerize with each other, probably via the GAF domain, and exist as components of high-molecular-mass protein complexes (Xie et al., 2006; Gao et al., 2008; Grefen et al., 2008; Chen et al., 2010). The results from those studies indicate that the ethylene response is negatively regulated by the cooperation of the five ethylene receptors. How the five ethylene receptors cooperate and the functional significance of the receptor cooperation remain to be addressed.

The results of this study show that ethylene responses were differentially repressed by the combination of a dominant, ethylene-insensitive receptor gene with a wild-type receptor gene. The dominant mutant receptor etr1-1 prevented ethylene responses primarily independent of cooperation with other wild-type receptors. In contrast, the repression of ethylene responses by the dominant, ethylene-insensitive *ers1-1*$^{(I62P)}$ was primarily dependent on *ETR1* or *EIN4* and differentially supported by the other three receptor genes (*ERS1*, *ETR2*, and *ERS2*) to lesser degrees. Among these three receptor genes, *ERS2* supported *ers1-1*$^{(I62P)}$ functions to a greater extent than did *ETR2*, whereas *ERS1* effects were marginal. The differential repression of ethylene responses by a combination of the dominant *ers1-1*$^{(I62P)}$ with wild-type receptor genes indicates that differential cooperation of ers1-1$^{(I62P)}$ and the corresponding receptors occurs. ERS1 does not contain the receiver domain; our data do not indicate that the presence of the receiver domain and the degree of cooperative receptor signaling are related. Notably, ETR1 substantially supported ers1-1$^{(I62P)}$ functions, whereas the ETR1-ERS1 association was relatively weak (Gao et al., 2008); thus, the degree of receptor cooperation and the strength of the receptor physical interaction are not necessarily related. This argument is in accordance with the implication that higher order receptor interactions are not stably preserved during the solubilization of receptor complexes (Chen et al., 2010).

The differential repression of ethylene responses by *ETR1*, *ERS1* (Liu et al., 2010), and their dominant ethylene-insensitive alleles, in combination with other receptor genes, may be a result of their relative receptor amounts, differential receptor efficacies or activity, receptor-specific interactions, or differential activation of CTR1 by these receptors or receptor complexes. The in vivo heterodimerization of the ethylene receptors and the formation of receptor complexes in Arabidopsis (Gao et al., 2008; Grefen et al., 2008; Chen et al., 2010) provide an explanation, on a molecular basis, for how these receptors may cooperate.

The relationship of receptor gene expression and receptor amount has been indicated previously, where EIN4 abundance is probably lowest among the five receptors (O'Malley et al., 2005; Gao et al., 2008). We showed that *EIN4* expression, and probably EIN4 level, was unaltered in *ein4 gers1-1*$^{(I62P)}$ and that *EIN4* alone was able to support *ers1-1*$^{(I62P)}$ functions to a large extent. The expression of *gers1-1*$^{(I62P)}$ in (*ERS2*) 4LOF largely prevented many aspects of the ethylene response, although *ERS2* expression was highly reduced. These results imply that a relatively low level of EIN4 or ERS2 is sufficient to support the ethylene insensitivity conferred by the dominant mutant receptor ers1-1$^{(I62P)}$ to a large extent. In contrast, *ERS1*/*ers1-1*$^{(I62P)}$ levels, and probably their protein amount, were highly induced in (*ERS1*)4LOF *gers1-1*$^{(I62P)}$, which exhibited a strong phenotype in various aspects of the

ethylene response. ETR2 undergoes bulk degradation at a high ethylene concentration (more than 100 μL L^{-1}), and the ligand-induced turnover can be prevented by an ethylene-insensitive receptor or the ethylene antagonist Ag(I) (Chen et al., 2007). The weaker effect of ETR2 on ers1-1$^{(I62P)}$ functions is unlikely to be the result of ETR2 turnover in response to ethylene, because ers1-1$^{(I62P)}$ is ethylene insensitive and presumably the ETR2 turnover is prevented. Collectively, these data favor the argument that the degree of ethylene-response repression by the dominant mutant receptor ers1-1$^{(I62P)}$ is strongly related to the identity of the receptor with which ers1-1$^{(I62P)}$ acts cooperatively. Our study, however, did not address the possible roles of receptor amount in ethylene signaling.

Redundant genes may exploit differential efficiencies, generated by divergence, to respond to fluctuating stimuli (Kafri et al., 2009). Currently, little is known about the nature of the receptor signal, and the receptor activity or efficacy is not biochemically measurable. Nevertheless, receptor efficacy can be evaluated from alteration of different aspects of the ethylene response. ETR1 shows a stronger association with CTR1 than ERS1, and the ETR2-CTR1 association is relatively weak (Clark et al., 1998; Cancel and Larsen, 2002). The association strength of each ethylene receptor member with CTR1 may have a role in differential CTR1 activation, by which ethylene responses can be repressed to different extents. Alternatively, each receptor may have a specific efficacy in CTR1 activation, which results in the differential repression of ethylene responses. (*ETR1*)4LOF exhibited the weakest ethylene response phenotype among the five receptor quadruple mutants, and (*ers1-2*)5LOF *getr1-1* was ethylene insensitive, which implied that ETR1 has the highest efficacy in the repression of ethylene responses. In contrast, ERS1 and its ethylene-insensitive mutant isoforms alone fail to repress constitutive ethylene responses via the activation of CTR1.

Synergistic (or superadditive) phenotypes refer to the phenotype alterations affected by genetically redundant mutations being much stronger than those

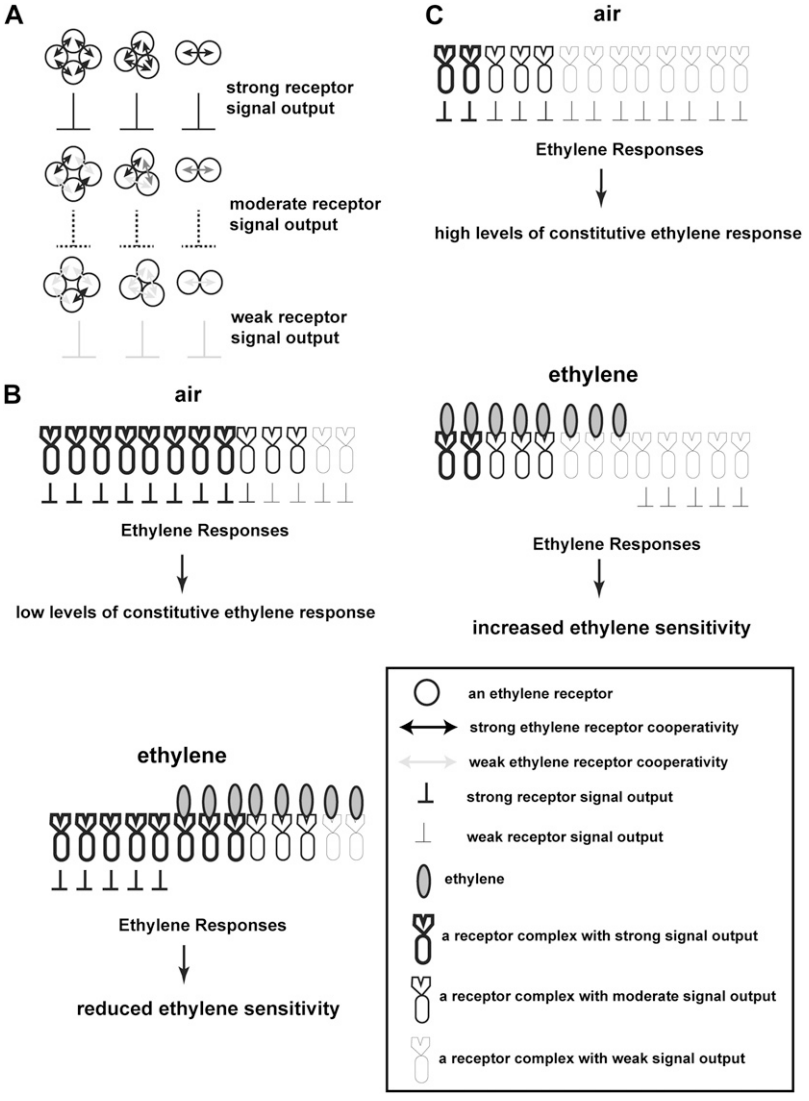

Figure 7. Model for the differential modulation of ethylene responses with multiple ethylene receptors. A, Diagrammatic illustration of ethylene receptor complexes that may consist of various receptor oligomers. Receptor members of a complex may differentially cooperate and mediate a gradient, from strong to weak, of receptor signal output. Shading differences indicate the receptor cooperativity of different levels. B and C, A cell or tissue may have mixed receptor complexes with different receptor signal output strengths. When the predominant receptor complexes can mediate strong signal output (B), constitutive ethylene responses will be suppressed to a greater extent than those that mediate weak signal output (C). Ethylene binding will remove the suppression, and ethylene responses can proceed. With the same ethylene concentration that does not saturate the ethylene receptors, a fraction of the receptor complexes will remain unbound and can suppress ethylene responses. Ethylene responses will be suppressed to a greater extent when the unbound receptor complexes predominantly mediate strong receptor signal output (B) and to a lesser extent when the unbound receptor complexes predominantly mediate weak receptor signal output.

caused by the additive effects of individual mutations (Pérez-Pérez et al., 2009). Synergistic phenotypes may occur when pathways are disturbed at a convergent node or when one mutation enhances the effects of another (i.e. the genes cooperatively act toward an outcome; Martienssen and Irish, 1999; Pérez-Pérez et al., 2009). In this study, the synergistic effects of *ETR1* and *EIN4* on *ers1*^{C65Y} functions were revealed from the results that showed that a lack of either gene did not significantly affect *ers1*^{C65Y} functions, whereas the lack of both genes almost abolished *ers1*^{C65Y} functions. Notably, the stronger effects of the *etr1* and *ein4* mutations than the additive effects of *etr1* and *ein4* on the ethylene response phenotype also indicate that ETR1 and EIN4 act synergistically with other wild-type receptors, so that the lack of both ETR1 and EIN4 results in strong growth inhibition. The *etr1* and *ers1* loss-of-function mutations also have synergistic effects on the ethylene response phenotype (Wang et al., 2003; Xie et al., 2006; Qu et al., 2007); thus, it is conceivable that the synergistic actions of different ethylene receptors play important roles in higher order receptor cooperation that differentially modulate different aspects of the ethylene response.

Although ethylene binding to ers1-1$^{(I62P)}$ and ers1^{C65Y} has never been investigated, the two mutations may convert ERS1 to an ethylene-insensitive state by different mechanisms. The protein conformations of ers1-1$^{(I62P)}$ and ers1^{C65Y} may differ so that each isoform may preferentially cooperate with specific receptors. Thus, a lack of the same receptors has different effects on the activity of these isoforms, which explains why the two isoforms differentially repressed ethylene responses in the same mutation background. ers1-1$^{(I62P)}$ and ers1^{C65Y} may represent ERS1 conformations that are changed by the mutations. It is conceivable that a wild-type ethylene receptor may undergo dynamic conformation changes that facilitate various differential cooperation with other ethylene receptors.

The results of this study imply that synergistic actions and higher order cooperation of ethylene receptors play important roles in the modulation of ethylene responses. Similar receptor cooperation is found in bacteria, where methyl-accepting chemotaxis protein (1-MCP) family receptors of high and low abundance, assembled in a higher order trimer of dimers, can signal cooperatively to enhance detection sensitivity in a five-log concentration range (Jasuja et al., 1999; Ames et al., 2002; Falke, 2002; Sourjik and Berg, 2002). We hypothesize that the ethylene receptor complexes, consisting of various receptor oligomers, may mediate a gradient, from strong to weak, of ethylene receptor signal output by which ethylene responses are differentially repressed, and thus a plant is induced to respond to a wide range of ethylene concentrations. Conceivably, the tissue-specific ethylene receptor compositions may facilitate differential receptor signal output, so that different plant tissues may exhibit specific ethylene responsiveness and ethylene responses can be modulated by alteration of the ethylene receptor composition (Fig. 7). This study provides explanations for why plants may use multiple ethylene receptors to regulate ethylene responses and reveals the important roles of wild-type ethylene receptors in supporting ethylene insensitivity conferred by a dominant, ethylene-insensitive receptor.

MATERIALS AND METHODS

Plant Materials

Arabidopsis (*Arabidopsis thaliana*) ethylene receptor mutants and transformation lines were obtained by genetic crosses or transformation and identified by genotyping (Xie et al., 2006; Liu et al., 2010). The growth conditions were described previously (Xie et al., 2006; Liu et al., 2010). Adult plants and light-grown seedlings were phenotyped at 4 weeks and 7 d post germination, respectively. (*ETR1*)4LOF, (*ERS1*)4LOF, (*ETR2*)4LOF, (*EIN4*)4LOF, and (*ERS2*)4LOF represent *ers1 etr2 ein4 ers2*, *etr1 etr2 ein4 ers2*, *etr1 ers1 ein4 ers2*, *etr1 ers1 etr2 ers2*, and *etr1 ers1 etr2 ein4*, respectively. Except for (*ETR1*)4LOF and (*ERS1*)4LOF, which were generated previously (Liu et al., 2010), (*ETR2*)4LOF, (*EIN4*)4LOF, and (*ERS2*)4LOF were obtained by genetic crosses as described below. In this study, the *ein4-7* allele was used and designated *ein4*.

Crossing of Transformation Lines

etr1 etr2 ein4 getr1-1 (previously designated *T:getr1-1 etr1 etr2 ein4*; Xie et al., 2006) was crossed with *etr2 ein4 ers2* to obtain *etr1 etr2 ein4 ers2 getr1-1*, designated (*ERS1*)4LOF *getr1-1*. The resulting (*ERS1*)4LOF *getr1-1* was next crossed with *ers1-2 etr2 ein4 ers2* [designated (*ETR1*)4LOF] to obtain *etr1 ers1-2 etr2 ein4 ers2 getr1-1*(+/−) (carrying a single copy of the *getr1-1* transgene) and *etr1 ers1-2 etr2 ein4 ers2 getr1-1* [designated (*ers1-2*)5LOF *getr1-1*]. (*ers1-2*)5LOF *getr1-1* was crossed with *ers1-3 etr2 ein4 ers2* to obtain *etr1 ers1-3 etr2 ein4 ers2 getr1-1*(+/−) and *etr1 ers1-3 etr2 ein4 ers2 getr1-1*. *gers1-1*$^{(I62P)}$ was transformed to *ETR1/etr1-7 ers1-2 etr2 ein4 ers2 getr1-1*(+/−); (*ers1-2*)5LOF *gers1-1*$^{(I62P)}$, *ETR1/etr1-7 ers1-2 etr2 ein4 ers2 gers1-1*$^{(I62P)}$, and (*ETR1*)4LOF *gers1-1*$^{(I62P)}$ were obtained after segregation of the *getr1-1* transgene and/or the *etr1* allele. *ETR1/etr1-7 ers1-2 etr2 ein4 ers2 gers1-1*$^{(I62P)}$ was crossed with *etr2 ein4 ers2* and *etr1 etr2 ers2* to obtain (*ERS1*)4LOF *gers1-1*$^{(I62P)}$ and (*EIN4*)4LOF *gers1-1*$^{(I62P)}$, respectively. *ETR1/etr1-7 ers1-2 etr2 ein4 ers2 gers1-1*$^{(I62P)}$ was crossed with *ers1 ein4 ers2* and *etr1 etr2 ein4* to obtain (*ETR2*)4LOF *gers1-1*$^{(I62P)}$ and (*ERS2*)4LOF *gers1-1*$^{(I62P)}$, respectively. *etr1 ers1-2 gers1-1*$^{(I62P)}$ and *etr1 ein4 gers1-1*$^{(I62P)}$ were obtained by transformation of *gers1-1*$^{(I62P)}$ to *etr1 ERS1/ers1-2* and *etr1 ein4*, respectively. *gers1*^{C65Y} was transformed to *etr1 ein4* to obtain *etr1 ein4 gers1*^{C65Y}. *etr1 ein4 gers1*^{C65Y} was crossed with Col-0 to obtain *gers1*^{C65Y} (Col-0), *etr1 gers1*^{C65Y}, and *ein4 gers1*^{C65Y}. (*ETR2*)4LOF, (*EIN4*)4LOF, and (*ERS2*)4LOF were obtained from the transformation quadruple mutants by segregation of the transgene. The genetic crosses are depicted in Figure 3B.

Gas Treatments and Analysis of the Ethylene Response Phenotype

Ethylene and 1-MCP concentrations were determined by gas chromatography with flame ionization detection (Agilent Technologies; 6890N Network GC System) as described previously (Zhang and Wen, 2010). For seed germination in the dark, the seeds were stratified at 4°C for 96 h and then transferred to 22°C for germination in the dark for 72 h in a temperature-controlled growth chamber. The seedling triple-response assay and the seedling hypocotyl measurement were as described previously (Xie et al., 2006; Liu et al., 2010). Treatment with 1-MCP (Rohm & Haas China) and ethylene (220 nL L^{-1} 1-MCP with 1 μL L^{-1} ethylene) was as described by Hall et al. (2000). For the measurement of ethylene-induced gene expression, the ethylene concentration was 10 μL L^{-1}. For the leaf senescence test, the ethylene concentration was 100 μL L^{-1}. The leaf chlorophyll content was determined as described previously (Zhang and Wen, 2010).

Transgenes

An *ers1-1*$^{(I62P)}$ fragment was generated by PCR from *ers1-1*$^{(I62P)}$ genomic DNA with the primer set ERS1 *Bam*HI-F (5′-CAGGATCCATGGAGTCATGC-

GATT-3′) and ERS1 *Sph*I-R (5′-ATGGCATGCATCGGTGTCCTCAT-3′). The resulting fragment was subcloned and confirmed by sequencing. An *ERS1* cDNA fragment, released by *Sph*I and *Xba*I, was ligated to the PCR-generated *ers1-1*$^{(I62P)}$ fragment to give rise to a full-length *ers1-1*$^{(I62P)}$ clone. The full-length *ers1-1*$^{(I62P)}$ clone was released by *Bam*HI and subcloned to a native *ERS1* promoter (Liu et al., 2010). All PCR-derived clones were confirmed by sequencing. The *ers1*^{C65Y} clone was constructed as follows. Primers ERS1 GAF-*Kpn*I-F (5′-TAGGTACCATGGAGTCATGCGATTGT3′) and ers1^{C65Y}-R (5′-ATGCGTAGCTCCATAGAGAATGATAA-3′) were used to generate the ers1^{C65Y} (1–207 bp) fragment; the C65Y mutation was included in the primer ers1^{C65Y}-R. ers1^{C65Y}-F (5′-TTATCATTCTCTATGGAGCTACGCAT-3′) and ERS1 geno-*Sph*I-R2 (5′-GAGATGATGGCATGCATCGGTGTCCT-3′) were used to generate *ers1*^{C65Y} (182–1,186 bp). The two PCR products were mixed to generate ers1^{C65Y} (1–1,186 bp) by the overlap extension PCR technique with the primer set ERS1 GAF-*Kpn*I-F and ERS1 geno-*Sph*I-R2. The resulting *ers1*^{C65Y} (1–1,186 bp) fragment was subcloned and sequenced. An *ERS1* cDNA fragment, released by *Kpn*I/*Sph*I, was ligated into the *ers1*^{C65Y} (1–1,186 bp) fragment, giving rise to a full-length *ers1*^{C65Y} clone. The full-length *ers1*^{C65Y} clone was subcloned to a native *ERS1* promoter.

RT-PCR

The fluorescence real-time qRT-PCR technique involved the use of the StepOne Real-Time PCR System (ABI) with the SYBR Premix Ex Taq real-time RT-PCR kit (Takara). Ubiquitin gene expression was used as an internal calibrator (Liu et al., 2010). The sequences for primer sets for each receptor gene were as described (Liu et al., 2010). The primers for *ERF1* were ERF-377-F (5′-TTTCTCGATGAGAGGGTC-3′) and ERF-606-R (5′-AAGCTCCTCAAGGTACTG-3′), and those for *SAG12* were SAG12-F (5′-TGAGGATGTCCCGGTTAATGAT-3′) and SAG12-R (5′-GATGATCCAATACTTTGATCCGTTAGT-3′). Each analysis was repeated three times with three independent biological repeats ($n = 3 \times 3$). For the measurement of ethylene-induced *ERF1* expression, plants were subjected to ethylene treatment for 4 h (Zhang and Wen, 2010); for the measurement of *SAG12* expression, plants were subjected to ethylene treatment for 36 h (Grbić, 2003). The primers for *ETR1* and *etr1-1* expression in an *etr1-7* background were ETR1-WT-F (5′-ATCTTATTAACTTATGG-3′) and ETR1-WT-R (5′-TGAGTTCGAATCAATCCCATTTCT-3′).

Immunoassays

The polyclonal antibodies for ERS1 (ERS1-Ab) were as described previously (Liu et al., 2010). Total protein was isolated as described by Wen et al. (1999) or with the use of TRIzol reagent (Invitrogen) in accordance with the manufacturer's instructions. In brief, plant tissue was homogenized in TRIzol reagent mixed with isopropanol, and total protein was collected by centrifugation. The resulting protein pellet was serially washed with 0.3 M guanidine hydrochloride in 95% ethanol. The resulting pellet was dissolved in 1% SDS and subjected to gel electrophoresis and immunoassay. ERS1 (or ers1-1^{I62P}) protein was detected by ERS1-Ab and Amersham ECL Plus Western Blotting Detection Reagents.

Supplemental Data

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

Supplemental Figure S1. Hypocotyl length of (*ETR1*)4LOF and (*ERS1*) 4LOF seedlings.

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