Ethylene Receptors Signal via a Noncanonical Pathway to Regulate Abscisic Acid Responses[1][OPEN]

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Ethylene is a gaseous plant hormone perceived by a family of receptors in Arabidopsis (*Arabidopsis thaliana*) including ETHYLENE RESPONSE1 (ETR1) and ETR2. Previously we showed that *etr1-6* loss-of-function plants germinate better and *etr2-3* loss-of-function plants germinate worse than wild-type under NaCl stress and in response to abscisic acid (ABA). In this study, we expanded these results by showing that ETR1 and ETR2 have contrasting roles in the control of germination under a variety of inhibitory conditions for seed germination such as treatment with KCl, $CuSO_4$, $ZnSO_4$, and ethanol. Pharmacological and molecular biology results support a model where ETR1 and ETR2 are indirectly affecting the expression of genes encoding ABA signaling proteins to affect ABA sensitivity. The receiver domain of ETR1 is involved in this function in germination under these conditions and controlling the expression of genes encoding ABA signaling proteins. Epistasis analysis demonstrated that these contrasting roles of ETR1 and ETR2 do not require the canonical ethylene signaling pathway. To explore the importance of receptor-protein interactions, we conducted yeast two-hybrid screens using the cytosolic domains of ETR1 and ETR2 as bait. Unique interacting partners with either ETR1 or ETR2 were identified. We focused on three of these proteins and confirmed the interactions with receptors. Loss of these proteins led to faster germination in response to ABA, showing that they are involved in ABA responses. Thus, ETR1 and ETR2 have both ethylene-dependent and -independent roles in plant cells that affect responses to ABA.

Ethylene is a phytohormone that affects the growth and development of plants and mediates plant stress responses (Mattoo and Suttle, 1991; Abeles et al., 1992). Many studies over the last several decades have identified constituents of the ethylene signaling pathway. This model proposes that activity of the receptors is reduced upon binding ethylene leading to reduced activity of a protein kinase, CONSTITUTIVE TRIPLE RESPONSE1 (CTR1; Kieber et al., 1993). Lower activity of CTR1 leads to a reduction in the phosphorylation of ETHYLENE INSENSITIVE2 (EIN2), causing a decrease in the ubiquitination of EIN2 and a rise in EIN2 protein levels; this allows for the proteolytic release of the C-terminal portion of the protein via an unidentified protease (Qiao et al., 2009, 2012; Chen et al., 2011; Ju et al., 2012; Wen et al., 2012). The EIN2 C-terminal portion modulates two transcription factors, EIN3 and EIN3-LIKE1, leading to the majority of ethylene responses (Chao et al., 1997; Solano et al., 1998; Alonso et al., 1999; Guo and Ecker, 2003; Yanagisawa et al., 2003; Binder et al., 2004a, 2004b; Gagne et al., 2004; Qiao et al., 2012).

Ethylene receptors are localized to the membrane of the endoplasmic reticulum (Chen et al., 2002; Ma et al., 2006; Grefen et al., 2008; Bisson et al., 2009). In Arabidopsis (*Arabidopsis thaliana*), there are five ethylene receptor isoforms known as ETHYLENE RESPONSE1 (ETR1), ETR2, ETHYLENE RESPONSE SENSOR1 (ERS1), ERS2, and EIN4 (Chang et al., 1993; Hua and Meyerowitz, 1998; Hua et al., 1998; Sakai et al., 1998; Gao et al., 2003). Based on sequence comparisons, the ethylene receptors fall into two subfamilies with ETR1 and ERS1 in subfamily 1 and ETR2, ERS2, and EIN4 in subfamily 2. All of the receptor isoforms contain an ethylene binding domain at the N terminus that is composed

[1] This project was supported by United States National Science Foundation (NSF) grants IOS-1254423 and MCB-1517032 to B.M.B.

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A.B., C.C., and B.M.B. designed the research; A.B., C.C., and J.C.F. performed research; A.B., S.P., C.C., T.H., and B.M.B. analyzed data; A.B. and B.M.B. wrote the paper.

[OPEN] Articles can be viewed without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.17.01321

of three membrane-spanning α-helices (Schaller and Bleecker, 1995; Schaller et al., 1995; Rodríguez et al., 1999). They all also contain a cytosolic portion with a GAF domain and a kinase domain. Three of the Arabidopsis receptors (ETR1, ETR2, and EIN4) contain a receiver domain at the C terminus. All five of the receptors have kinase activity with ETR1 containing His kinase activity, ETR2, ERS2, and EIN4 containing Ser/Thr kinase activity, and ERS1 displaying both depending on the assay conditions (Gamble et al., 2002; Moussatche and Klee, 2004; Bisson and Groth, 2010).

Although the receptors overlap in the control of many traits, they also have nonredundant roles in the regulation of various traits (Shakeel et al., 2013). For instance, in Arabidopsis, the subfamily-1 receptors have a larger role in the control of growth than the subfamily-2 receptors and this is not simply a matter of different levels of the receptors (O'Malley and Bleecker, 2003; Wang et al., 2003; Qu et al., 2007). It is possible that these differences are in part due to different interactors. For instance, ETR1, and to a lesser extent ERS1, are affected by REVERSION TO ETHYLENE SENSITIVITY1, whereas, the other three receptors are not (Resnick et al., 2006; Deslauriers et al., 2015). A second example is that ETR1, ETR2, and EIN4 (receptors containing a receiver domain) are important for growth recovery after ethylene removal, but ERS1 and ERS2 are not (Binder et al., 2004a, 2004b). Additionally, in Arabidopsis, silver ions, which are well known to inhibit the ethylene receptors (Beyer, 1976), act predominantly via the ETR1 receptor (McDaniel and Binder, 2012). Surprisingly, there are also some phenotypes where the different ethylene receptor isoforms have contrasting roles. For example, ethylene-stimulated nutational bending of Arabidopsis hypocotyls requires the ETR1 receptor, whereas, the other four receptor isoforms inhibit nutations (Binder et al., 2006; Kim et al., 2011). Another example is that ERS1 and ETR1 have opposite roles in the control of growth (Liu et al., 2010). Recently, we found that ETR1 and ETR2 have contrasting roles in the control of Arabidopsis seed germination in darkness and during NaCl stress in the light where ETR1 hinders seed germination and ETR2 increases seed germination (Wilson et al., 2014a, 2014b; Bakshi et al., 2015). EIN4 has a smaller role similar to ETR1 in the control of seed germination during these stresses, whereas ERS1 and ERS2 have no measurable roles in regulating this trait (Wilson et al., 2014a, 2014b). These contrasting roles seem to be independent of changes in ethylene biosynthesis or sensitivity, and likely involves a change in abscisic acid (ABA) responsiveness (Wilson et al., 2014a, 2014b). These data are not explained by existing models of ethylene signal transduction, and point to unique roles for the ETR1 and ETR2 receptors in the control of ABA signal transduction.

A large body of research has led to models for ABA signaling (Cutler et al., 2010; Umezawa et al., 2010; Yang et al., 2017). In these models, ABA binds to PYRABACTIN RESISTANCE1 (PYR1) and PYR1-like (PYL) receptors leading to inactivation of some type 2C protein phosphatases from group A (PP2CA; Fujii and Zhu, 2009; Fujita et al., 2009; Ma et al., 2009; Melcher et al., 2009; Miyazono et al., 2009; Nishimura et al., 2009; Park et al., 2009). Most of the PP2CAs involved in ABA signaling act as negative regulators of the pathway so that when inactivated by the receptors, ABA signaling proceeds. This occurs by the activation of SUC NONFERMENTING1-RELATED PROTEIN KINASE2s (SnRK2s), which in turn activate the ABA-RESPONSIVE ELEMENTS BINDING FACTOR (ABF) transcription factors causing an alteration in the transcription of ABA-responsive genes (Mustilli et al., 2002; Yoshida et al., 2006a, 2006b; Boudsocq et al., 2007; Nakashima et al., 2009; Umezawa et al., 2009; Vlad et al., 2009; Soon et al., 2012; Waadt et al., 2015). Several PP2CAs are positive regulators of the pathway. ABA also leads to changes in anion channels (Fujii et al., 2009; Geiger et al., 2009; Xue et al., 2011; Brandt et al., 2012). The gene transcripts encoding for many of these proteins in the ABA signaling pathway are up-regulated by ABA and various stresses (Chan, 2012).

Prior research suggests models where the ethylene receptors have roles independent of the canonical ethylene signaling pathway (Gamble et al., 1998; Beaudoin et al., 2000; Desikan et al., 2005; Binder et al., 2006; Resnick et al., 2006; Dong et al., 2008, 2010; Qiu et al., 2012; Kim et al., 2013; Wilson et al., 2014a, 2014b; Bakshi et al., 2015), which may underlie these observations. Establishing the mechanisms for noncanonical receptor signaling is of interest across kingdoms because noncanonical signal transduction has been observed in other signal transduction pathways of animals and bacteria (Federle and Bassler, 2003; Jenkins, 2009; Miller and McCrea, 2010; Chen et al., 2015). To understand the mechanism for the contrasting roles of ETR1 and ETR2 in germination, we determined that these receptors are predominantly affecting ABA signaling to alter germination in response to many stresses. Unexpectedly, we found that the role of ETR1 and ETR2 in seed germination occurs independently of the canonical ethylene signal transduction pathway. Because of this, we used yeast two-hybrid to screen for interacting partners with ETR1 and ETR2 and identified many putative nonoverlapping interaction proteins that may underlie their contrasting roles in the control of seed germination. We confirmed three of these interactions, and showed that loss of any of these three proteins led to altered germination in response to ABA. Thus, ETR1 and ETR2 signal via a noncanonical pathway to affect ABA signal transduction.

RESULTS

ETR1 and ETR2 Have Opposite Roles in the Control of Seed Germination under Various Inhibitory Conditions

We have previously shown that ETR1, and to a lesser extent EIN4, inhibits and ETR2 promotes seed germination under NaCl stress and in darkness (Wilson et al., 2014a, 2014b). We aimed to know if these opposite roles extended to other conditions that delay Arabidopsis seed germination such as stress caused by other salts,

heavy metals, ethanol, and short-day conditions (Shinomura et al., 1994; Hirayama et al., 2004; Li et al., 2005; Wang et al., 2007, 2008). To examine this, we measured the germination time-courses for wild-type, *etr1-6*, *etr1-7*, *ein4-4*, and *etr2-3* seeds under control conditions and conditions that introduced one of the following variables: 150 mM NaCl, 150 mM KCl, 100 μM $CuSO_4$, 300 μM $ZnSO_4$, 100 mM ethanol, and short-day conditions (8 h light/16 h dark). Seed germination was considered to be complete upon rupture of the testa. As a control for sulfate ions in the $CuSO_4$ and $ZnSO_4$ conditions, we examined the effect of 100 μM and 300 μM Na_2SO_4 on the time-course of germination. We calculated the time for 50% germination under each of these conditions. Consistent with our prior results (Wilson et al., 2014a, 2014b), NaCl delayed seed germination of all three seed lines and *etr1-6*, *etr1-7*, and *ein4-4* mutant plants germinated faster than wild-type and *etr2-3* mutant plants germinated slower under this condition (Fig. 1A; Supplemental Fig. S1). Germination was delayed in wild-type seeds to a lesser extent by KCl, $CuSO_4$, $ZnSO_4$, and ethanol compared to NaCl at the concentrations used. In all these conditions, the *etr1-6*, *etr1-7*, and *ein4-4* seeds germinated faster and the *etr2-3* seed germinated slower than wild-type. Consistent with our prior study using NaCl (Wilson et al., 2014a, 2014b), there was a smaller effect of the *ein4-4* mutation compared to loss of *ETR1*. Treating seeds with either 100 μM or 300 μM $NaSO_4$ had no measurable effect on seed germination, indicating that the delay in germination caused by $CuSO_4$ and $ZnSO_4$ is due to the heavy metals, not the sulfate ions. In short-day conditions, the *etr1-6*, *etr1-7*, and *ein4-4* mutant plants germinated slightly faster than wild-type seeds and the *etr2-3* mutant plants slightly slower than wild-type seeds. But these differences were not statistically significant ($P > 0.05$).

We have previously concluded that ETR1 and ETR2 probably affects sensitivity of seeds to ABA to alter seed germination (Wilson et al., 2014a, 2014b). Abiotic stress conditions are known to cause an increase in ABA biosynthesis leading to inhibition of germination (Vishwakarma et al., 2017). We therefore treated germinating seeds with 100 μM norflurazon (NF) to inhibit the biosynthesis of ABA in the germinating seeds. Treatment with NF drastically reduced or eliminated the differences in seed germination seen among wild-type, *etr1-6*, *etr1-7*, *ein4-4*, and *etr2-3* in NaCl, KCl, $CuSO_4$, $ZnSO_4$, and ethanol (Fig. 1B; Supplemental Fig. S2). Application of NF had no significant effect on seed germination under control conditions. This indicates that ETR1, EIN4, and ETR2 are likely to also be affecting ABA under these various conditions that inhibit seed germination.

The Receiver Domain of ETR1 Is Required for its Role in Seed Germination under Various Inhibitory Conditions

We have previously shown that the receiver domain of ETR1 is required for its inhibitory role on seed germination during NaCl stress, but not in darkness (Wilson et al., 2014a, 2014b). Additionally, several amino acids in the receiver domain are critical for normal ETR1 function related to germination on NaCl, but not in the control of other traits such as growth inhibition and ethylene-stimulated nutations (Bakshi et al., 2015). To determine whether the receiver domain is required for the control of seed germination by ETR1 under other inhibitory conditions, we examined germination time-courses of *etr1-6;etr2-3;ein4-4* triple-mutant plants lacking ethylene receptor isoforms with a receiver domain, and this triple mutant transformed with cDNA for the full-length *ETR1* transgene (*cETR1*) or a truncated *ETR1* transgene lacking the receiver domain (*cetr1-ΔR*). Consistent with our previous results (Wilson et al., 2014a, 2014b), the *etr1-6;etr2-3;ein4-4* triple mutant plants germinated faster than wild-type seeds under NaCl stress; the *cETR1* transgene caused germination to be slower and comparable to wild type, whereas the *cetr1-ΔR* transgene failed to delay germination (Fig. 2A; Supplemental Fig. S3). We observed a similar pattern with KCl, $CuSO_4$, $ZnSO_4$, and ethanol, suggesting that ETR1 functions similarly in all of these conditions. Thus, the receiver domain is required for the function of ETR1 in regulating seed germination under these inhibitory conditions.

In a prior study (Bakshi et al., 2015), we found that transforming the *etr1-6;etr2-3;ein4-4* triple-mutant plants with genomic DNA encoding for an *ETR1* transgene containing a *D659A* mutation (D659A) in the receiver domain resulted in ETR1 having a reduced function in the control of germination in seeds treated with NaCl, because it poorly rescued the time-course of germination to wild-type level. By contrast, an *ETR1* transgene with an *E666A* mutation (E666A) causes ETR1 to be hyperfunctional for this trait so that germination on NaCl is slower than the triple mutant transformed with the wild-type genomic *ETR1* (*gETR1*) transgene. Both of these mutant transgenes have normal function in controlling other traits (Bakshi et al., 2015). We conducted seed germination time-course experiments with these transformants, and observed a similar pattern for all of these inhibitory conditions where D659A was not functional and E666A was hyperfunctional in the conditions tested (Fig. 2A; Supplemental Fig. S3). This further supports the idea that ETR1 has a similar role in the control of germination under these various conditions that inhibit germination.

Unlike the above results, the receiver domain of ETR1 is not needed for ETR1 to inhibit germination in darkness (Wilson et al., 2014a, 2014b). We were therefore curious to know if the D659A or E666A mutations affected germination in darkness. Germination in darkness is very poor and wild-type seeds failed to reach 50% germination 7 d after planting (Supplemental Fig. S4), consistent with our prior study (Wilson et al., 2014a, 2014b). Therefore, we examined the extent of germination of *etr1-6;etr2-3;ein4-4* triple-mutant plants and transformants 7 d after planting and maintained in darkness. We found, like our prior study, that wild-type seeds germinated very poorly, that *etr1-6;etr2-3;ein4-4* triple-mutant plants germinated to

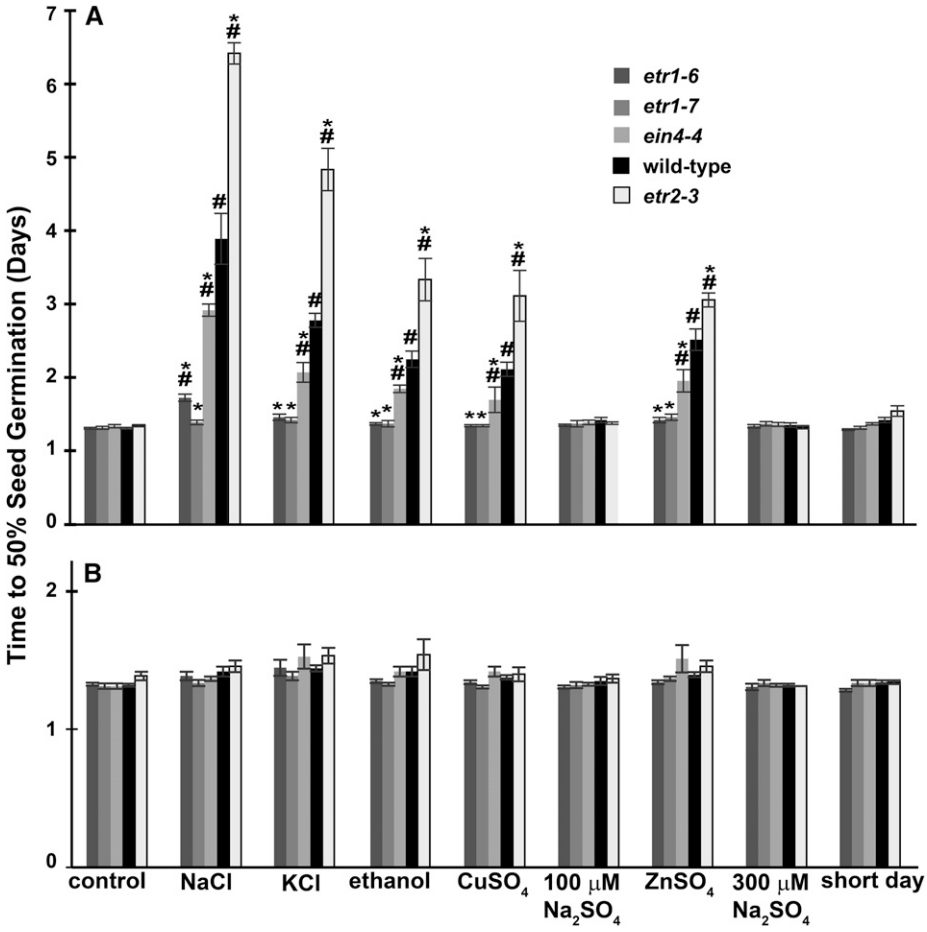

Figure 1. ETR1 and ETR2 have contrasting roles on seed germination under a range of inhibitory conditions. Germination time-courses for wild-type, *etr1-6*, *etr1-7*, *ein4-4*, and *etr2-3* seeds were conducted as described in the "Materials and Methods" and the times for 50% of seeds to germinate were calculated. Germination experiments were conducted in the (A) absence or (B) presence of 100 μM NF to inhibit ABA biosynthesis. Conditions used were: control (using standard conditions described in the "Materials and Methods"), 150 mM NaCl, 150 mM KCl, 100 mM ethanol, 100 μM $CuSO_4$, 100 μM $NaSO_4$, 300 μM $ZnSO_4$, 300 $NaSO_4$, and short days (8-h light:16-h dark). Data represents the average ± SD. Data were analyzed using two-way ANOVA and Tukey's multiple comparisons test. (*) Statistically different from wild type in that condition. (#) Statistically different from untreated seeds of that seed line ($P < 0.05$).

100% and that both *cETR1* and *cetr1-ΔR* transgenes reversed this, causing the seeds to germinate poorly (Fig. 2B). Similarly, the *D659A* or *E666A* mutant transgenes caused the triple-mutant plants to germinate poorly. All seed lines reached 100% germination within 2.5 d of being transferred from darkness to long-day conditions as previously reported (Wilson et al., 2014a, 2014b), showing that all seeds were viable. Thus, unlike the above stress conditions, the ETR1 receiver domain is not needed nor influences germination in darkness.

ETR1, EIN4, and ETR2 Affect Responsiveness to ABA

The above results, as well as our previous findings (Wilson et al., 2014a, 2014b), suggest that ETR1, EIN4, and ETR2 are affecting ABA sensitivity or levels or both. In the control of seed germination, we previously showed that *etr1-6* seeds are less sensitive to ABA, and *etr2-3* seeds are more sensitive to ABA compared to wild-type (Wilson et al., 2014a, 2014b). However, it is unclear if ABA synthesis may also be affected in these mutants. To distinguish between these possibilities, we examined the effect of 1 μM ABA in the presence and absence of 100 μM NF on seed germination. We posited that if the ETR1, EIN4, and ETR2 receptors are only affecting ABA sensitivity, then NF should have no effect on germination time-courses under these conditions. However, if these receptors are affecting biosynthesis of ABA, then NF should eliminate or reduce germination differences. Consistent with Wilson et al. (2014a, 2014b), in the presence of ABA, the *etr1-6* mutant plants germinated faster than wild-type seeds and the *etr2-3* mutant plants germinated slower than wild-type seeds (Fig. 3A). Similar to the results examining germination under stress, both the *etr1-7* and *ein4-4* seeds also germinated faster than wild-type in the presence of ABA. The application of NF had no measurable effect on this, supporting the idea that ETR1, EIN4, and ETR2 mainly affect sensitivity to ABA.

To confirm that ABA responsiveness is altered, we used real-time qRT-PCR to examine the transcript levels of several ABA-responsive genes including *CRUCIFERIN1* (*CRA1*) encoding for a seed storage protein, *RESPONSIVE TO ABA18* (*RAB18*) encoding for a dehydrin protein, a stress-responsive protein called *KIN1*, and *RESPONSIVE TO DESICATION 29A* (*RD29A*) encoding for a Leu zipper transcription factor (Pang et al., 1988; Lång and Palva, 1992; Chan, 2012; Gliwicka et al., 2012). We compared transcript levels of these genes in seeds germinated for 2 d in the presence or absence of 1 μM ABA. Because ETR1 and ETR2 are having the largest effects on germination,

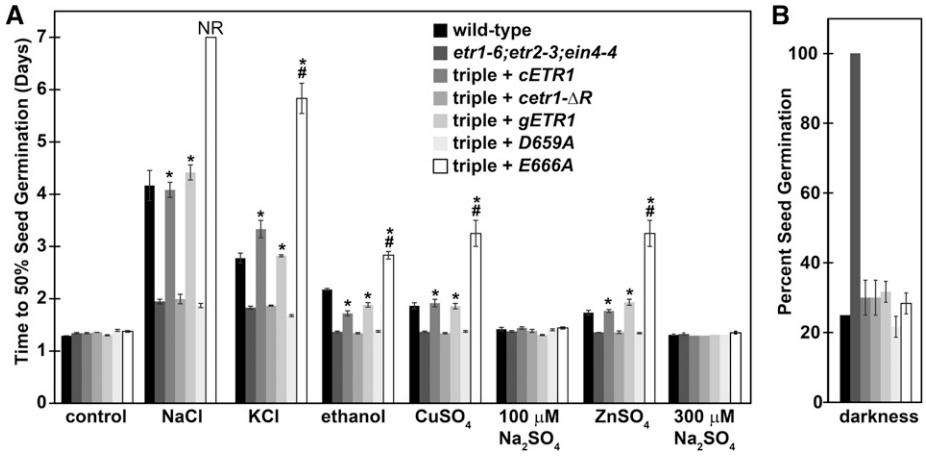

Figure 2. The rapid germination of *etr1*;*etr2*;*ein4* triple mutant plants under various inhibitory conditions is differentially rescued by truncated and *etr1* receiver domain mutants. A, Germination time-courses were determined for *etr1-6*;*etr2-3*;*ein4-4* triple mutant plants and these triple mutants were transformed with a cDNA for full-length *ETR1* (*cETR1*) or a truncated *ETR1* lacking the receiver domain (*cetr1-ΔR*) or genomic DNA for full-length wild-type *ETR1* (*gETR1*), a *D659A* mutant, or an *E666A* mutant. The times for 50% germination were then calculated. Conditions used were control (using standard conditions described in the "Materials and Methods"), 150 mM NaCl, 150 mM KCl, 100 mM ethanol, 100 μM $CuSO_4$, 100 μM $NaSO_4$, 300 μM $ZnSO_4$, and 300 μM $NaSO_4$. B, Percent seed germination of seeds kept in darkness 7 d after planting. For both panels, data are the average ± SD. Data were analyzed using two-way ANOVA and Tukey's multiple comparisons test. NR, never reached 50% germination in the time-course of the experiment. (*) Denotes the *etr1-6*;*etr2-3*;*ein4-4* triple mutant transformed with the indicated transgene is statistically different from the triple mutant. (#) The transformant is statistically slower than triple mutant transformed with full-length *ETR1* transgene ($P < 0.05$).

we compared the transcript levels in *etr1-6* and *etr2-3* mutant seeds. Consistent with Bakshi et al. (2015), application of ABA caused *CRA1* and *RAB18* transcript levels to rise in wild-type seeds (Fig. 3B). Similarly, ABA also increased the transcript abundance of *KIN1* and *RD29A* in wild-type seeds. In the absence of exogenous ABA, loss of either ETR1 or ETR2 had no statistically significant effect on the transcript levels of these four genes. However, when ABA was applied, the induction for these gene transcripts was larger in *etr2-3* seeds and generally reduced in *etr1-6* seeds compared to wild type. Interestingly, we previously showed that ABA has little or no effect on the transcript levels of *CRA1* and *RAB18* in *etr1-6;etr2-3;ein4-4* triple-mutant plants (Bakshi et al., 2015). Transformation of the triple-mutant plants with *gETR1* rescued this response; by contrast, transformation with D659A failed to rescue this induction, and transformation with E666A led to an enhanced induction of these genes that correlates with the effects of these point mutants on germination (Bakshi et al., 2015). Together, these data show that ETR1 and ETR2 are affecting ABA signal transduction oppositely and this is influenced by the ETR1 receiver domain.

We wished to determine what aspects of ABA signaling are altered in these mutants. The gene transcripts encoding for many of ABA signaling proteins are up-regulated by ABA and various stresses (Chan, 2012). We therefore used real-time qRT-PCR to study the transcript abundance of 25 genes that encode for proteins involved in ABA signaling (Fig. 4). This included genes that encode for the ABA receptors *PYR1*, *PYL1*, *PYL5*, *PYL7*, and *PYL9*, the PP2CAs *ABA INSENSTIVE1* (*ABI1*), *ABI2*, *ABA-HYPERSENSITIVE GERMINATION1* (*AHG1*), *AHG3*, *HOMOLOGY TO ABI1* (*HAB1*), *HAB2*, *HIGHLY ABA-INDUCED1* (*HAI1*), *HAI2*, and *HAI3*, the SnRK2s *SnRK2.2*, *SnRK2.3*, *SnRK2.6*, and *SnRK2.10*, the ABFs *ABF1*, *ABF2*, *ABF3*, *ABF3*, and *ABI5*, and the *SLOW ANION CHANNEL-ASSOCIATED1* (*SLAC1*) and *SLAC1-HOMOLOG3* (*SLAH3*) ion channels.

In the absence of exogenous ABA, loss of either ETR1 or ETR2 generally had no effect on the transcript abundance of most of these genes (Fig. 4). The exceptions were *PYR1* and *PYL1*, where loss of either ETR1 or ETR2 led to a 3-to 5-fold decrease in the transcript abundance of these genes that encode for receptors. There was also a slight effect on the transcript levels of *HAB2*, where *etr1-6* seeds had slightly higher levels than *etr2-3* seeds and wild-type seeds had intermediate levels in the absence of added ABA.

In wild-type seeds, application of ABA caused an increase in the transcript abundance of *PYL7*, *PYL9*, *ABI2*, *AHG3*, *HAB1*, *HAB2*, *HAI2*, *HAI3*, *ABF1*, *ABF2*, *ABF4*, *ABI5*, and *SLAC1* and a decrease in *PYR1* and *PYL1* transcript levels ($P < 0.05$; Fig. 4). Application of ABA to wild-type seeds caused little or no change in the transcript levels of *ABI1*, *AHG1*, *HAI1*, *SnRK*20.2, *SnRK2.3*, *SnRK2.6*, *SnRK2.10*, *ABF3*, and *SLAH3*, or else the changes observed were below the statistical cutoff we used ($P > 0.05$).

In most cases, ABA caused the smallest increase in *etr1-6* mutant plants and the largest increase in *etr2-3* mutant plants with wild-type seeds having intermediate

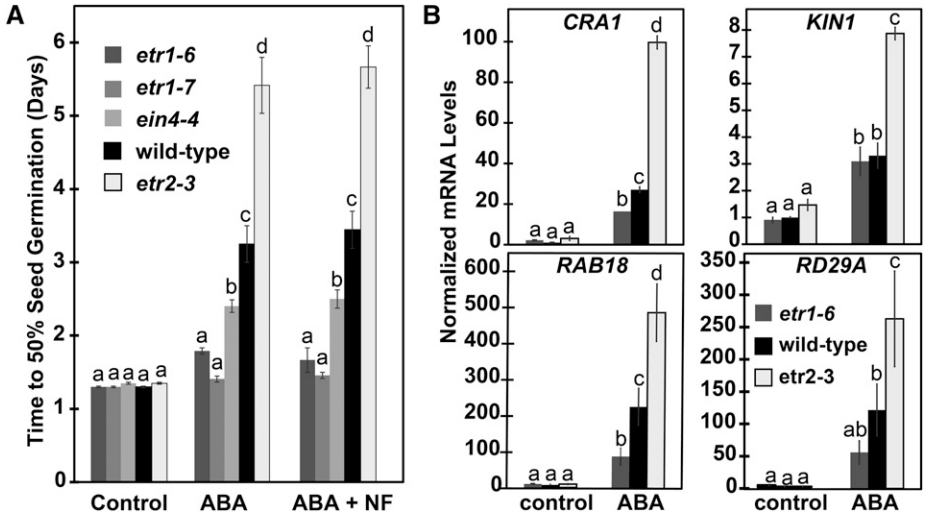

Figure 3. ETR1 and EIN4 affect responses to ABA oppositely from ETR2. A, Germination time-courses of wild-type, *etr1-6*, *etr1-7*, *ein4-4*, and *etr2-3* seeds in response to 1 μM ABA and 1 μM ABA plus 100 μM NF to block ABA biosynthesis were conducted and the times for 50% seed germination were determined. Data represents the average ± SD. B, The transcript abundance of *RAB18*, *CRA1*, *KIN1*, and *RD29A* were measured in wild-type, *etr1-6*, and *etr2-3* seeds using real-time qRT-PCR. Seeds were germinated for 2 d in the presence or absence of 1 μM ABA and mRNA extracted. Data were normalized to the levels of At3g12210 in each seed line to calculate the relative transcript level for each gene. These were then normalized to levels of the transcript in untreated wild-type seeds. The average ± SE for two biological replicates with three technical replicates each is shown. For both panels, 0.01% (v/v) ethanol was used as a solvent control. Data were analyzed using a two-way ANOVA and Tukey's multiple comparisons test. In each panel, the different letters indicate significant difference ($P < 0.05$).

responses (Fig. 4). This pattern was seen with *PYL7*, *PYL9*, *HAI1*, *HAI2*, *HAI3*, *SnRK2.2*, *SnRK2.10*, *ABF1*, *ABF2*, *ABF3*, *ABF4*, *ABI5*, and *SLAC1*. By contrast, application of ABA caused the largest change in the transcript levels of six gene transcripts encoding the PP2CAs *ABI1*, *ABI2*, *AHG1*, *AHG3*, *HAB1*, and *HAB2* in *etr1-6* seeds and smallest change in *etr2-3* seeds. Contrary to the genes mentioned above, these PP2CAs inhibit ABA signaling (Gosti et al., 1999; Merlot et al., 2001; Leonhardt et al., 2004; Saez et al., 2004; Kuhn et al., 2006; Yoshida et al., 2006a, 2006b; Nishimura et al., 2007; Rubio et al., 2009). Application of ABA had little or no effect on the transcript levels of *PYR1*, *PYL1*, *PYL5*, *SnRK2.3*, *SnRK2.6*, and *SLAH3* in the *etr1-6* and *etr2-3* mutant seeds.

Together, these data support a model where, in the presence of ABA, ETR1 and ETR2 lead to opposite changes in the transcript abundance of many genes that encode for proteins involved in ABA signal transduction leading to changes in ABA sensitivity.

The Receiver Domain of ETR1 Is Involved in the Induction of ABA Signaling Genes by ABA

We were also interested to know if the ETR1 receiver domain was important for the induction of these gene transcripts. We therefore examined the transcript abundance of three of the genes that encode for ABA signaling components in the in *etr1-6;etr2-3;ein4-4* triple-mutant plants and the triple mutants transformed with the *gETR1*, *D659A*, or *E666A* transgenes. We chose one positive (*PYL7*) and two negative (*ABI1*, *AHG1*) regulators of ABA signaling that showed a clear difference in the *etr1-6* versus *etr2-3* mutants in the presence of ABA (Fig. 4).

In the absence of exogenous ABA, there were no statistically significant differences ($P > 0.05$) in the transcript levels of the three genes in wild-type versus the triple-mutant seeds (Fig. 5). However, under this condition, transformation of the triple mutant with *D659A* led to an increase in transcript levels of *ABI1* and *AHG1* compared to the other seed lines.

In the presence of exogenous ABA, transformation of the triple mutant with the *D659A* transgene led to comparable levels of *PYL7*, *ABI1*, and *AHG1* compared to the triple mutants, indicating this transgene was minimally functional. By contrast, transformation of the triple mutant with the *E666A* transgene led to an increase of *PYL7* transcript abundance compared to the *gETR1* transformant and lower levels of *AHG1* transcript abundance compared to the *gETR1* transformant. Additionally, the levels of *PYL7* gene transcript was higher in the E666A seed line compared to all the other seed lines including wild-type. By contrast, the levels of *ABI1* and *AHG1* transcripts were lower than wild type in the E666A seed line. Thus, in the presence of ABA, D659A is causing lower levels of the positive ABA regulator and higher levels of the negative regulators correlating with less ABA signaling and faster germination of this transformant. By contrast, E666A is having the opposite effect, which correlates with higher levels of ABA signaling and slower germination of this transformant.

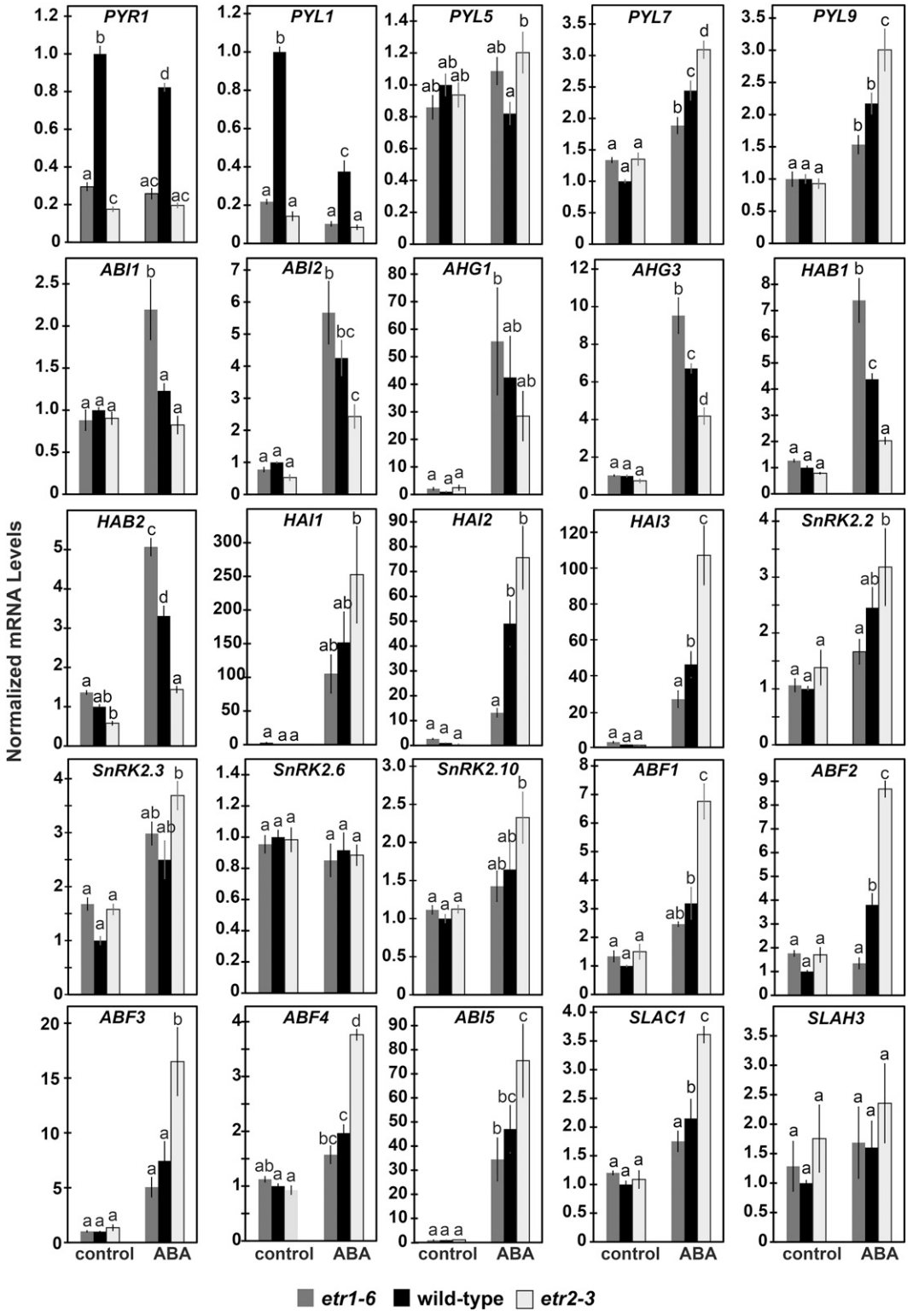

Figure 4. ETR1 and ETR2 oppositely affect the transcript abundance of many genes encoding for ABA signaling proteins. The transcript levels of genes in germinating seeds encoding for proteins in the ABA signal transduction pathway were analyzed with real-time qRT-PCR as described in Figure 3. The average ± SE for two to three biological replicates with three technical replicates each is shown. In all panels, 0.01% (v/v) ethanol was used as a solvent control. Data were analyzed using a two-way ANOVA and Tukey's multiple comparisons test. In each panel, different letters indicate significant difference ($P < 0.05$).

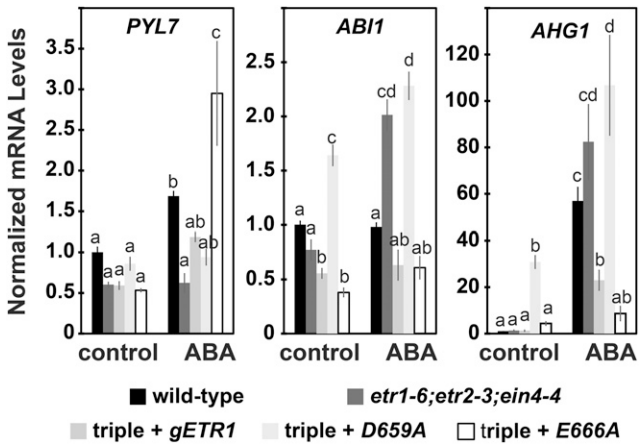

Figure 5. The *D659A* and *E666A* mutant transgenes oppositely affect the transcript abundance of genes encoding for ABA signaling proteins. The transcript levels of selected genes encoding for ABA signaling proteins were determined for *etr1-6;etr2-3;ein4-4* triple mutant plants and these triple mutants transformed with genomic DNA for full-length wild-type *ETR1* (*gETR1*), a *D659A* mutant, or an *E666A* mutant. For comparison, data for wild-type seeds are shown. The transcript levels of genes in germinating seeds encoding for proteins in the ABA signal transduction pathway were analyzed with real-time qRT-PCR as described in Figure 3. The average ± SE for two biological replicates with three technical replicates each is shown. Data were analyzed using a two-way ANOVA and Tukey's multiple comparisons test. In each panel, different letters indicate significant difference ($P < 0.05$).

These results are consistent with the ETR1 receiver domain being involved in the changes in the levels of genes encoding for ABA signaling genes.

ABA Affects the Transcript Levels of *ETR1*, *EIN4*, and *ETR2*, But Not *ERS1* and *ERS2*

Using epistasis analysis, we have previously posited that ETR2 signals via ETR1 and EIN4, to control germination in response to stress or application of ABA (Wilson et al., 2014a, 2014b). In this model, the ETR1 and EIN4 receptors function in parallel to stimulate ABA signal transduction, with ETR1 having the larger role in this. The function of ETR2 is to inhibit both receptors. It is unclear how ETR2 is having this effect. It is known that stress and application of ABA can affect the transcript levels of the ethylene receptors in various plant species (Zhao and Schaller, 2004; Martín-Rodríguez et al., 2011; Mou et al., 2016). We were therefore curious to know if application of ABA altered the transcript levels of the five ethylene receptors, and whether loss of either *ETR1* or *ETR2* affected ABA-induced changes in the other receptors.

In the absence of exogenous ABA, loss of *ETR1* or *ETR2* had no measurable effect on transcript levels of the remaining four receptor isoforms (Fig. 6A). Application of 1 μM ABA caused an increase in the transcript levels of *ETR1* and *EIN4* in wild-type seeds. Interestingly, ABA had no effect on *EIN4* transcript abundance in the *etr1-6* seeds, suggesting that ABA signaling is reduced to a level where it does not affect *EIN4* transcription. By contrast, the ABA-stimulated increase in both *ETR1* and *EIN4* transcript abundance was larger in *etr2-3* mutant seeds. Application of ABA caused no measurable change in the levels of *ETR2*, *ERS1*, or *ERS2* transcripts in wild-type seeds. However, loss of *ETR1* resulted in ABA treatment causing an increase in *ETR2* transcript abundance. Application of ABA had no measurable effect on the transcript abundance of either *ERS1* or *ERS2* in the *etr1-6* and *etr2-3* seeds. These data suggest that output from the ABA signal transduction pathway is affecting the levels of the *ETR1*, *EIN4*, and *ETR2* transcripts. In the case of *ETR1* and *EIN4* transcript levels, ETR2 is diminishing, but not completely blocking the ABA-stimulated increase in transcript abundance. By contrast, ETR1 is preventing the ABA-stimulated increase in *ETR2* transcript abundance so that the increase is only seen when ETR1 is removed.

These results lead to a model for how ETR2 inhibits ETR1 and EIN4 by preventing accumulation of *ETR1* and *EIN4* transcripts (Fig. 6B). These data also suggest that ETR1 is negatively affecting the accumulation of *ETR2* transcript in the presence of ABA, leading to feedback on this circuit to cause more signaling from ETR1 due to higher *ETR1* transcript abundance. The ERS1 and ERS2 receptors have no apparent role in regulating ABA to affect seed germination because loss of either *ERS1* or *ERS2* has no effect on seed germination in response to NaCl stress (Wilson et al., 2014a, 2014b) and ABA fails to cause changes in *ERS1* and *ERS2* transcript levels.

ETR1 and ETR2 Signal Independently of CTR1 to Control Seed Germination

Ethylene is known to stimulate Arabidopsis seed germination (Bleecker et al., 1988). However, we previously showed that the effects of the *etr1-6* and *etr2-3* mutations on germination are likely to not involve ethylene signaling because *etr2-3* seeds are more sensitive to application of ethylene than wild-type and *etr1-6* seeds (Wilson et al., 2014a, 2014b). This is opposite to what is predicted if ethylene is involved. Additionally, ethylene biosynthesis is minimally affected in the mutants compared to wild-type during germination under NaCl stress conditions. Current models of ethylene signaling posit that all receptor signaling occurs via CTR1 and EIN2. However, various studies have provided evidence that the receptors may signal via alternate pathways in addition to the canonical pathway (Gamble et al., 1998; Beaudoin et al., 2000; Desikan et al., 2005; Binder et al., 2006; Qiu et al., 2012; Kim et al., 2013; Wilson et al., 2014a, 2014b; Bakshi et al., 2015).

To more thoroughly test whether ETR1 and ETR2 are affecting seed germination independently of the canonical ethylene signaling pathway, we generated *etr1-6;ctr1-2* and *etr2-3;ctr1-2* double mutant plants. It has previously been demonstrated that *ctr1* mutant

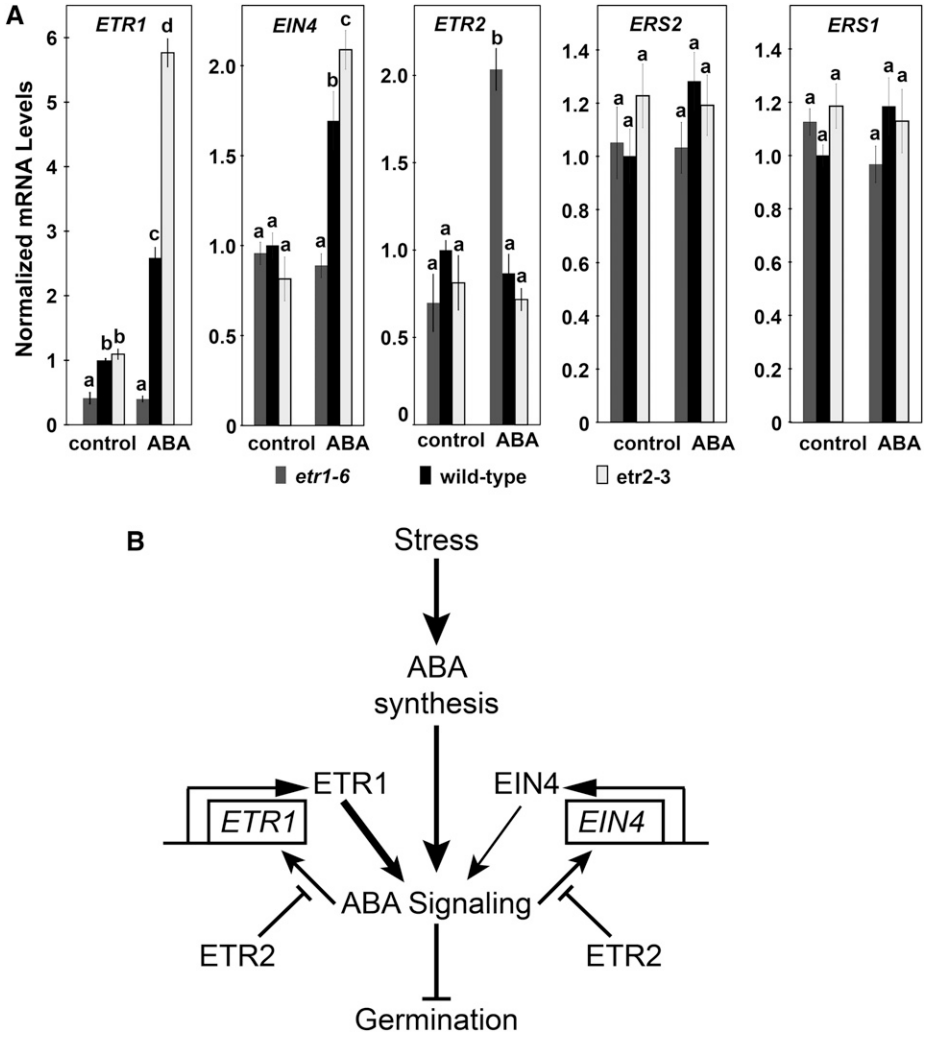

Figure 6. ETR1 and ETR2 affect ABA-induced changes in *ETR1*, *EIN4*, and *ETR2*, but not *ERS1* and *ERS2* transcripts. A, The transcript levels of the five ethylene receptor isoforms in germinating seeds were analyzed with real-time qRT-PCR as described in Figure 3. The average ± SE for two to three biological replicates with three technical replicates each is shown. In all panels, 0.01% (v/v) ethanol was used as a solvent control. Data were analyzed using the two-way ANOVA and Tukey's multiple comparisons test, and in each panel the different letters indicate significant difference ($P < 0.05$). B, Model of roles of ETR1, EIN4, and ETR2 in the control of ABA signaling during seed germination. In this model, it is proposed that various stresses increase ABA levels. Previous epistasis indicates that in the presence of ABA, ETR2 inhibits ETR1 and EIN4, which act in parallel to enhance ABA signaling (Wilson et al., 2014a, 2014b). Results here indicate that ETR2 inhibits ETR1 and EIN4 by reducing, but not eliminating, ABA-induced increases in *ETR1* and *EIN4* transcription. Not shown here, we also found that ETR1 suppresses ABA-induced changes in *ETR2* transcript abundance. It is possible that in the presence of ABA, EIN4 is also affecting transcript abundance of *ETR2*, but this has not yet been studied. The width of the arrows from ETR1 and EIN4 denote the relative signaling strength affecting ABA signaling. It is unknown whether these are direct effects on ABA signaling or happening via intermediaries.

plants germinate better in the presence of salt than wild-type seeds (Lin et al., 2012). Therefore, we predicted that if ETR1 and ETR2 are signaling via the canonical signaling pathway to control ABA sensitivity, in the presence of ABA, the double mutant plants containing *ctr1-2* should germinate similarly to *ctr1-2* single mutant plants. Double mutants with phenotypes that diverge from these predictions would support a model invoking signaling via a noncanonical pathway.

We generated crosses between either *etr1-6* or *etr2-3* and *ctr1-2*. We only obtained one cross of these because these crosses were very stunted and generated few seeds. Each double mutant was grown to the F4 generation. We used PCR to confirm that the double-mutant plants were homozygous for either *etr1-6* or *etr2-3* (Supplemental Fig. S5, A and B). When grown in the dark, Arabidopsis seedlings have long hypocotyls and treatment with ethylene results in short hypocotyls (Bleecker et al., 1988; Guzmán and Ecker, 1990). Using this assay, we confirmed that double mutant plants containing *ctr1-2* had a constitutive growth inhibition response when grown in ethylene-free air (Supplemental Fig. S5C)

similar to the findings of Kieber et al. (1993). Consistent with prior studies (Hua and Meyerowitz, 1998; Cancel and Larsen, 2002), the *etr1-6* mutant plants were slightly shorter than wild type in air and had a slightly stronger growth inhibition response upon addition of ethylene (Supplemental Fig. S5C).

In the absence of exogenous ABA, all seed lines had similar germination time-courses and times for 50% germination (Fig. 7A; Supplemental Fig. S6). In the presence of 1 μM ABA, the *ctr1-2* seeds germinated faster than wild-type seeds with a germination time-course slightly slower than *etr1-6* seeds (Fig. 7B; Supplemental Fig. S6). Interestingly, the *etr1-6;ctr1-2* mutant plants germinated faster than either the *etr1-6* or *ctr1-2* single mutant plants and the *etr2-3;ctr1-2* mutant plants gave a germination time-course intermediate between the single mutant parents. This additive behavior between the receptors and CTR1 suggest that the receptors are signaling, at least in part, independently of CTR1 to affect seed germination.

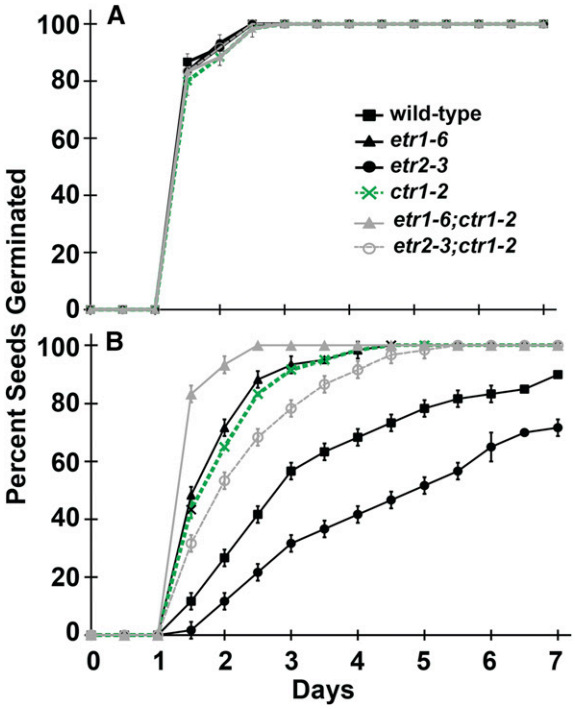

Figure 7. ETR1 and ETR2 function independently of the canonical ethylene signaling pathway to alter response to ABA. Double *etr1-6; ctr1-2* and *etr2-3;ctr1-2* mutant plants were generated and physiologically evaluated. For comparison, wild-type and *etr1-6, etr2-3,* and *ctr1-2* single mutant plants were included. Seed germination time-courses were conducted in the (A) absence and (B) presence of 1 μM ABA.

ETR1 and ETR2 Have Both Overlapping and Nonoverlapping Interaction Partners

The above results raise the possibility that ETR1 and ETR2 are involved in unidentified protein interactions to affect ABA signaling. To study this, we used yeast two-hybrid analysis to screen for interacting partners where we made bait constructs encoding for the cytosolic portion of each receptor. For ETR1, this included expressing amino acids 127 through 738 ($ETR1^{127\text{-}738}$) and for ETR2, amino acids 157 through 776 ($ETR2^{157\text{-}776}$), which included the GAF, kinase, and receiver domain of each but eliminated the N-terminal portion containing the transmembrane α-helices (Fig. 8A). Each construct was fused to the GAL4 DNA binding domain of pGBKT7 vector and then introduced into the yeast (*Saccharomyces cerevisiae*) strain Y187 to generate the bait strain. These were then used to screen cDNA prey libraries generated in yeast strain AH109 as a fusion to the GAL4 activation domain of the pGADT7 vector (Hewezi et al., 2008). A total of 52 possible interaction partners for $ETR1^{127\text{-}738}$ and 37 for $ETR2^{157\text{-}776}$ was identified; there were six that overlapped for both (Supplemental Table S1). A gene ontogeny (GO) classification analysis showed that more than half were related to stress responses including abiotic stresses, water stress, cold stress, and salt and osmotic stress (Fig. 8B). To map possible tissue coexpression of these with ETR1 or ETR2, we used the Multiple Experiment Viewer (http://mev.tm4.org/) to construct a heatmap of gene coexpression patterns in various tissues such as embryos, floral buds, flowers, hypocotyls, leaves, roots, shoot apical meristem, seedlings, and whole plants (Fig. 8C). From this information, we generated a gene coexpression map where we show all the interactions uncovered, as well as those that are predicted to occur in root tissue (Fig. 8D). It was interesting to note that the majority of interacting pairs are coexpressed in only one organ/tissue with most coexpressed in the roots. In a few cases, the putative interacting partners coexpress in two or, to a much lesser extent, in three tissues. The network contained 83 nodes and 87 edges representing the interacting combinations. However, two that were identified as potentially interacting with ETR1 (At4g18250, At5g37665) and two that were identified as possibly interacting with ETR2 (EF1α, FAH) appear to not be coexpressed in any tissue with their respective receptor partner. One putative interacting partner (At5g06380) that was identified as potentially interacting with both ETR1 and ETR2 appears to only be coexpressed with ETR1.

Analysis of Selected Protein Interactors

We next selected some of the potentially interesting interacting protein candidates for further analysis. For this, we focused on the DROUGHT-INDUCED19 (DI19) transcription factor, a β-glucosidase called LONG ER BODY (LEB), and the RESPONSIVE TO DEHYDRATION21A (RD21A) protease identified in the yeast two-hybrid screen. All three of these proteins are documented to be involved in stress responses or response to ABA and are predicted to be expressed in either the cytosol or endoplasmic reticulum. It is interesting to note that out of these three proteins, DI19 interacts with CPK11, which encodes for a Ca^{2+}-dependent, calmodulin-independent protein kinase that functions as a positive regulator of

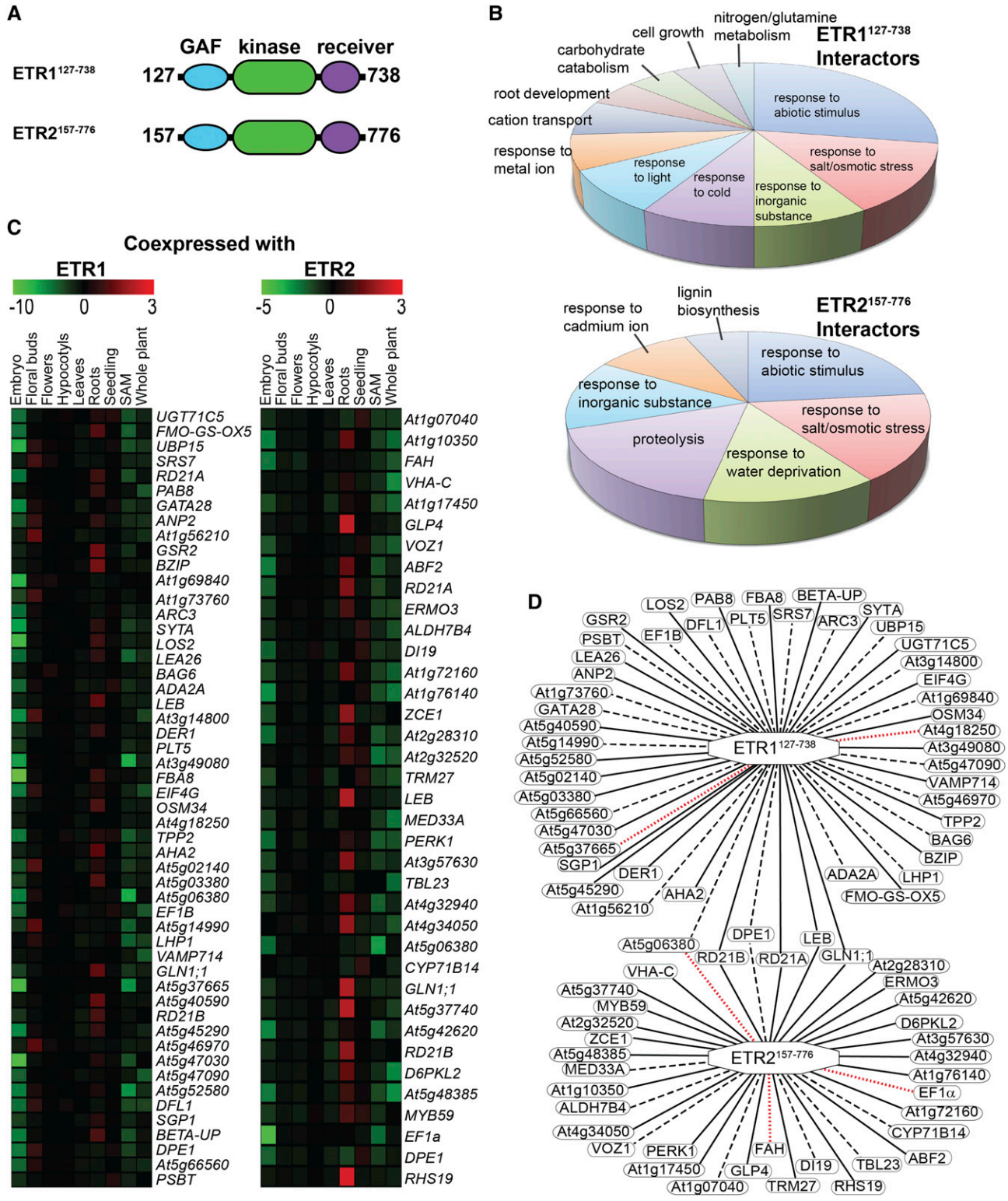

Figure 8. Yeast two-hybrid screen using the cytosolic domains of ETR1 and ETR2 reveals putative overlapping and nonoverlapping interaction partners. A yeast two-hybrid screen was carried out using the cytosolic portion of ETR1 (ETR1$^{127\text{-}738}$) or ETR2 (ETR2$^{157\text{-}776}$) as described in the "Materials and Methods". A, Diagram of cytosolic portions of ETR1 and ETR2 used for yeast two-hybrid screen. Numbers represent the amino acids included in these constructs. B, GO categorization of proteins identified in this screen as interacting with ETR1$^{127\text{-}738}$ or ETR2$^{157\text{-}776}$. GO enrichment analysis was carried out using DAVID (https://david.ncifcrf.gov/). Only GO categories with a false discovery rate value < 0.05 were included. C, Heatmap showing coexpression patterns for

ABA signaling pathway by phosphorylating ABA-responsive element binding protein factors ABF1 and ABF4 (Hayashi et al., 2001; Milla et al., 2006; Ma and Bohnert, 2007; Sherameti et al., 2008; Liu et al., 2013; Qin et al., 2014; Song et al., 2016).

To further test whether the identified proteins interacted with ETR1 and ETR2, we used yeast cotransformation assays where we cotransformed yeast (strain AH109) with a full-length cDNA encoding one of the identified targets and either $ETR1^{127\text{-}738}$ or $ETR2^{157\text{-}776}$. The full-length sequence encoding the individual interacting partners was cloned in a prey vector (pGADT7), whereas the C-terminal coding sequences of *ETR1* or *ETR2* were cloned into a bait vector (pGBKT7). Subsequently, yeast AH109 cells were cotransformed with the bait and prey vectors and potential interactions were visualized by differential growth on the nonselective synthetic dropout (SD) medium (SD/-Leu/-Trp) and on the selective medium (SD/-Leu/-Trp/-His/-Ade; Fig. 9A). Growth on the selective medium confirmed the yeast two-hybrid results that showed that RD21A and LEB interact with both ETR1 and ETR2, whereas DI19 only interacts with ETR2. Serial dilutions of yeast cotransformed cells were used to measure the strength of the interaction and we observed interactions with 1:100 dilutions.

To confirm the protein-protein interactions in planta, bimolecular fluorescence complementation (BiFC) assays were conducted, where we used the full-length coding sequences of the receptors fused to the N-terminal half of a yellow fluorescent protein gene (*nEYFP*), whereas the full-length clones of *DI19*, *RD21A*, or *LEB* were fused to the C-terminal half of the yellow fluorescent protein gene (*cEYFP*; Fig. 9B). The different combinations between nEYFP and cEYFP fusions were coexpressed in *Nicotiana benthamiana* cells via biolistic bombardment. As expected, negative controls gave no YFP signal. Consistent with the yeast two-hybrid screen and yeast cotransformation analysis, we found that DI19 only interacted with ETR2, whereas LEB and RD21A interacted with both receptors by reconstituting the fluorescent YFP in the transformed *N. benthamiana* cells. The DI19 and ETR1 interacting combination yielded no YFP fluorescence. The interactions of LEB and RD21A with ETR1 gave a weaker signal than what was observed with ETR2. Together, these data indicate that LEB and RD21A interact with both receptors and DI19 interacts with ETR2 both in yeast and in planta.

We wanted to know if these proteins are involved in ABA-induced changes in germination. We therefore measured the seed germination time-courses of two loss-of-function alleles each for DI19, LEB, and RD21A. These included the *pyk10-1* and *leb-1* mutants for LEB that have previously been characterized (Nagano et al., 2008, 2009), the SALK 065256 and SALK 090550 lines with mutations in RD21A, and the SALK 119971 and SALK 063827 lines with mutations in DI19. The SALK lines were confirmed to be homozygous using PCR as described in the "Materials and Methods". In these mutants, germination time-courses and time for 50% germination were similar to wild-type seeds in the absence of exogenous ABA (Fig. 9C; Supplemental Fig. S7). By contrast, loss of any one of these proteins led to faster germination than wild type in response to ABA. The time for 50% germination of these mutant plants in the presence of ABA was slightly slower than what was observed for the *etr1-6* mutant plants. These data indicate these proteins are involved in the control of seed germination in response to ABA.

We were curious to know if loss of either *ETR1* or *ETR2* affected the transcript abundance of *DI10*, *RD21A*, or *LEB*. In the absence of ABA, loss of these receptors had no measurable effect on the transcript abundance of *DI19* or *LEB* (Fig. 9D). By contrast, loss of either receptor caused a reduction in the transcript levels of *RD21A* in the absence of ABA. Application of ABA to wild-type seeds caused an increase of *DI19* transcript, a decrease in *RD21A* transcript, and no change in *LEB* transcript. The increase in *DI19* transcript was lost in *etr1-6* seeds and enhanced in *etr2-3* seeds. Similarly, loss of *ETR2* caused a larger increase in *LEB* transcript abundance upon application of ABA; however, loss of *ETR1* had no effect on *LEB* transcript levels. Loss of either receptor had no effect on transcript abundance of *RD21A* in the presence of ABA. Thus, under certain conditions the ETR1 and ETR2 receptors are affecting the transcript abundance of these genes that encode for interacting partners.

DISCUSSION

The signal transduction pathway for ethylene has been well studied. Nonetheless, many questions remain, including understanding the function of the ethylene receptors. This is complicated by a growing body of evidence indicating that the ethylene receptors have both overlapping and nonoverlapping roles (Shakeel et al., 2013). We previously reported that ETR1 and ETR2 have contrasting roles in the control of seed germination under NaCl stress and in response to ABA, and posited that these contrasting roles were likely independent of ethylene (Wilson et al., 2014a, 2014b). Here we demonstrated that ETR1 and ETR2 have contrasting roles under other conditions that

Figure 8. (*Continued.*)
ETR1 and ETR2 interacting proteins. Red indicates the protein has highly correlated gene coexpression with the receptor and green represents highly anticorrelated coexpression. D, Gene coexpression map of the ETR1 and ETR2 interacting partners. Solid edges represent proteins with significantly correlated expression profiles with the receptor in roots. Dashed edges represent proteins with significantly correlated coexpression profiles in at least one tissue, but not roots. Dotted red edges represent no significant correlation in the coexpression profile between the receptor and the gene in question.

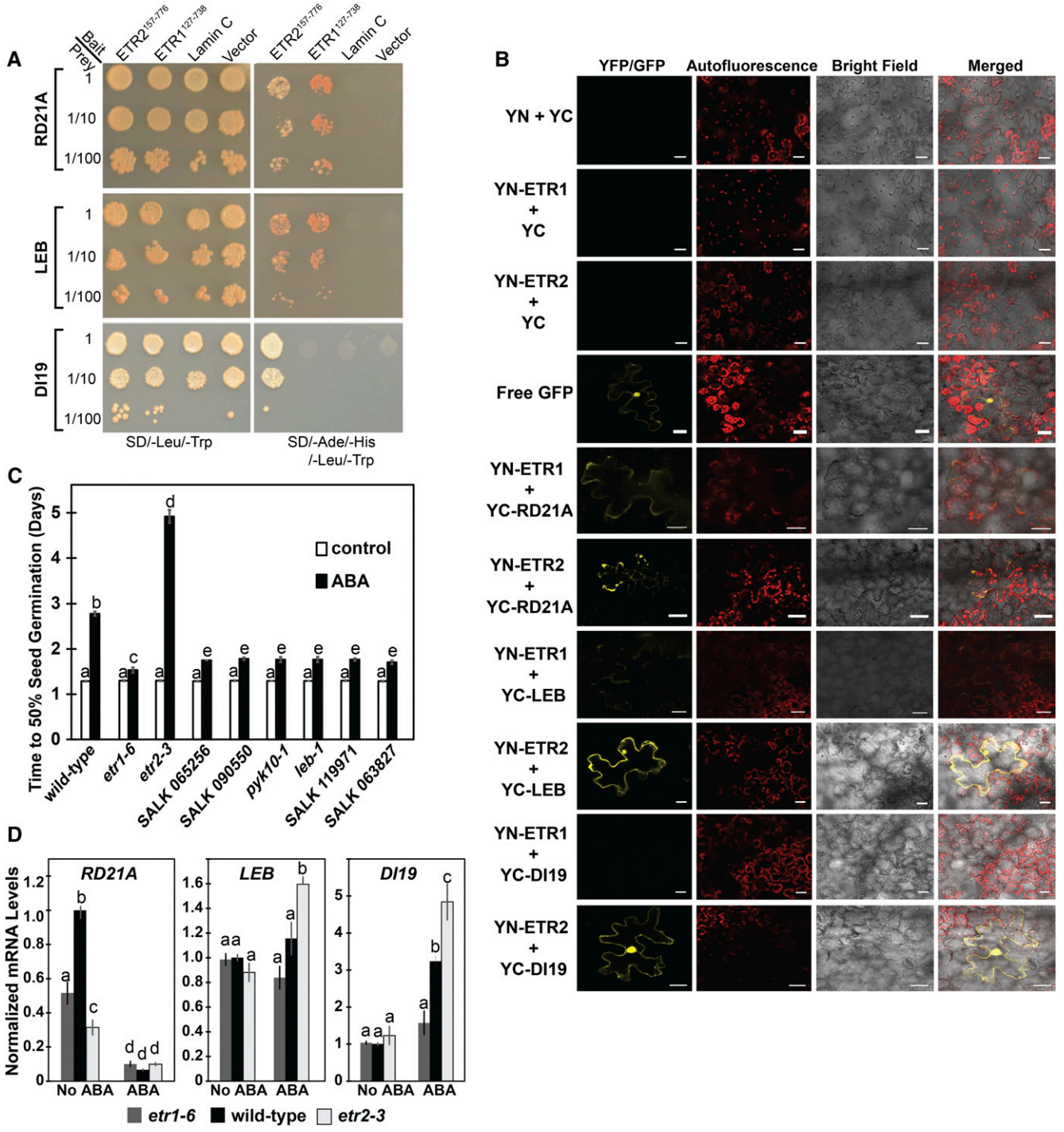

Figure 9. Three interaction partners affect seed germination in response to ABA. A, Cotransformation analysis was carried out as described in the "Materials and Methods". Yeast strain AH109 was cotransformed with full-length coding sequences of the selected interacting partners cloned in the prey vector (pGADT7) and C-terminal coding sequences of ETR1 ($ETR1^{127-738}$) or ETR2 ($ETR2^{157-776}$) cloned in the bait vector (pGBKT7). Protein-protein interactions were visualized by differential growth on the nonselective synthetic drop-out medium (SD/-Leu/-Trp; left) and on the selective medium (SD/- Leu/-Trp/-His/-Ade; right). B, BiFC in *N. benthamiana* was carried out with the full-length clones of *ETR1* or *ETR2* fused to YN and the full-length clones of the indicated proteins fused to the YC as described in the "Materials and Methods". RD21A, LEB, and DI19 were labeled at their N terminus with YC. ETR1 and ETR2 were labeled at their N terminus (YN-ETR1 and YN-ETR2, respectively) for interaction studies with LEB and RD21A and at their C terminus (ETR1-YN, ETR2-YN) for experiments with DI19. YN+YC, YN-ETR1+YC, and YN-ETR2+YC were included as negative controls and free GFP as a positive control. YFP or GFP fluorescence, chlorophyll autofluorescence, and bright field images were acquired and merged. Scale bar is 25 μm. C, Time for 50% germination of mutants

inhibit germination. The receiver domain of ETR1 has been implicated as being important for subfunctionalization of this receptor in other traits such as growth recovery after removal of ethylene, ethylene-stimulated nutations, and germination under NaCl stress (Binder et al., 2006; Wilson et al., 2014a, 2014b; Bakshi et al., 2015). The ETR1 receiver domain was also important for the control of germination under most of the additional stress conditions used in this study, suggesting a common mechanism for the control of germination by ETR1 under these conditions. The exception to this was that, consistent with a prior study (Wilson et al., 2014a, 2014b), the receiver domain of ETR1 is not required for ETR1 to affect germination in darkness.

We believe that ABA is involved in mediating the contrasting roles of ETR1 and ETR2 in germination under many stress conditions because ABA is known to be part of abiotic stress responses in plants (Vishwakarma et al., 2017) and NF eliminated germination differences under these stress conditions. Application of ABA caused the largest delay in the seed germination of *etr2-3* mutant plants and the smallest delay in *etr1-6* mutant plants, and this effect was unaffected by the application of NF to block ABA biosynthesis. This suggests that ETR1 and ETR2 are predominantly affecting ABA sensitivity, rather than ABA biosynthesis. Additional support for this is that ABA caused a smaller increase in the transcript levels of several ABA-responsive genes in the *etr1-6* mutant plants compared to *etr2-3* mutant plants. Unexpectedly, in the presence of ABA, both ETR1 and ETR2 affected the transcript abundance of many genes that encode for proteins in the ABA signaling pathway. Where such an effect was seen, in most cases the lowest gene transcript levels were seen in the *etr1-6* seeds and the highest in the *etr2-3* seeds. This included genes for several receptors (*PYL7* and *PYL9*), PP2CAs (*HAI1*, *HAI2*, and *HAI3*), SnRK2s (*SnRK2.2* and *SnRK2.10*), and ABFs (*ABF1*, *ABF2*, *ABF3*, *ABF4*, and *ABI5*). Because these genes are positive regulators of ABA signaling (Zhang et al., 2004; Fujii et al., 2007, 2011; Fujita et al., 2009; Bhaskara et al., 2012), the lower levels observed in the *etr1-6* seeds correlate with the smaller ABA responses we observe in this mutant during seed germination. Conversely, the higher levels of these genes in the *etr2-3* mutant plants correlate with the larger ABA responses we observed with *etr2-3*. The transcript abundance of six PP2CAs (*ABI1*, *ABI2*, *AHG1*, *AHG2*, *HAB1*, and *HAB2*) showed the opposite pattern with the highest transcript abundance in the *etr1-6* seeds and the lowest transcript abundance in the *etr2-3* seeds. Because these are negative regulators of ABA signaling (Leonhardt et al., 2004; Saez et al., 2004; Kuhn et al., 2006; Nishimura et al., 2007; Rubio et al., 2009; Antoni et al., 2012), this also correlates with the ABA responses we see in the *etr1-6* and *etr2-3* mutant plants. In the presence of ABA, the E666A mutation in ETR1 led to a larger induction and the D659A mutation to a smaller induction of ABA-responsive genes (Bakshi et al., 2015). Similarly, these mutations led to alterations in ABA-induced changes of gene transcripts for ABA signaling proteins that correlate with the changes these mutations have on germination during stress. Thus, ETR1 and ETR2 are having a widespread effect on transcript abundance of genes that encode for ABA signaling proteins and this is likely causing changes in ABA responsiveness. In the case of ETR1, this effect involves the receiver domain.

Using epistasis analysis among ETR1, ETR2, and EIN4, we previously generated a model where ETR1 and EIN4 inhibit seed germination by stimulating ABA signaling and ETR2 inhibits both receptors (Wilson et al., 2014a, 2014b). We found that ABA increased the transcript levels of *ETR1* and *EIN4*, but not *ETR2*, *ERS1*, or *ERS2* in wild-type seeds. The observation that loss of ETR2 leads to higher *ETR1* and *EIN4* transcript levels in the presence of ABA supports a model where ETR2 inhibits ETR1 and EIN4 via regulation of transcription (Fig. 6B). Interestingly, ABA caused an increase in *ETR2* transcript levels in *etr1-6* seeds, suggesting that ABA can affect both receptors, but that ETR1 inhibits this effect on *ETR2* overcoming the effect of ABA. These data suggest that there is feedback from ABA signaling to alter both receptors, and there is reciprocal regulation between ETR1 and ETR2 where each is negatively affecting the transcript levels of the other under these conditions (Fig. 6B). The role of the interacting proteins identified in this study remains unknown. We demonstrate that loss of *LEB*, *RD21A*, or *DI19* leads to reduced ABA responsiveness and faster seed germination similar to *etr1* loss-of-function mutants. However, the mechanism by which their interaction with ethylene receptors is important to affect germination is unknown. It is also likely that loss of other interactors will have an *etr2* loss-of-function phenotype where the mutants are more responsive to ABA and germinate more slowly. A simple genetic working model (Supplemental Fig. S8) illustrates how these proteins might interact to control ABA responsiveness. This opens up the possibility for future research using epistasis analysis between the ethylene receptors and proteins of interest to delineate the pathway(s) involved in affecting the ABA signaling pathway.

Figure 9. (*Continued.*)
for RD21A (SALK 065256, SALK 090550), LEB (*pyk10-1*, *leb-1*), and DI19 (SALK 119971, SALK 063827). Seeds were germinated in the absence or presence of 1 μM ABA as indicated, and the percent germination was determined every 12 h for 7 d and the time for 50% seed germination was calculated. For comparison, the germination of wild-type, *etr1-6*, and *etr2-3* seeds is shown. Data represents the average ± SD. D, The transcript levels of the *RD21A*, *LEB*, and *DI19* in germinating seeds were analyzed with real-time qRT-PCR as described in Figure 3. The average ± SE for two to three biological replicates with three technical replicates each is shown. In (C) and (D), days were analyzed using a two-way ANOVA and Tukey's multiple comparisons test. Different letters indicate significant difference ($P < 0.05$).

The biochemical outputs of ETR1 and ETR2 remain unknown. In the case of ETR1, signaling to inhibit seed germination under inhibitory stress conditions or in response to ABA requires the D659 amino acid residue in the receiver domain. This amino acid residue is thought to be the target of phosphorelay from the ETR1 His kinase (Gamble et al., 1998). However, neither this residue, nor the entire receiver domain, are required for ethylene signaling to control most traits (Gamble et al., 2002; Binder et al., 2004a, 2004b; Kim et al., 2011; Hall et al., 2012; Bakshi et al., 2015). Similarly, ETR1 His kinase activity is not required for ethylene signaling (Wang et al., 2003; Qu et al., 2007). This suggests that ETR1 His kinase activity and phosphotransfer have alternative roles outside of ethylene signal transduction. Because ABA signaling involves changes in the phosphorylation of various proteins (Cutler et al., 2010; Umezawa et al., 2010; Yang et al., 2017), it is possible that one such role for the ETR1 His kinase, and perhaps the ETR2 Ser/Thr kinase, is to modulate ABA signaling. Support for this is the report that Ser/Thr kinase activity in the NTHK1 (for *N. tabacum* HIS KINASE1) subfamily 2 ethylene receptor affects the sensitivity of tobacco (*N. tabacum*) to NaCl stress (Chen et al., 2009). It remains to be determined if ABA sensitivity is also altered in NTHK1 mutant plants.

All five ethylene receptors in Arabidopsis have been documented to physically interact with CTR1 (Clark et al., 1998; Cancel and Larsen, 2002; Gao et al., 2003; Bisson et al., 2009; Bisson and Groth, 2010) and most models suggest that all signaling from these receptors requires CTR1. However, ETR1 and ETR2 have opposite roles for seed germination in response to ABA. We previously showed that these contrasting roles do not correlate with biosynthesis of ethylene or sensitivity to ethylene in these mutants (Wilson et al., 2014a, 2014b). Therefore, a likely model is that there is an alternative, noncanonical signaling pathway from these two ethylene receptors that is independent of CTR1. Our epistasis analysis between receptor loss-of-function mutants and CTR1 support this model. Intermediate seed germination phenotypes in response to ABA were observed in the *etr1-6;ctr1-2* and *etr2-3;ctr1-2* as predicted if signaling is occurring, at least partially, independently of CTR1.

Receptor signaling independent of CTR1 raises the question of what proteins are involved in this alternative pathway. Our yeast two-hybrid analyses show that there are numerous potential candidates to examine because there were many nonoverlapping receptor-protein interactions identified. The fact that many of these potential interacting partners are annotated as stress-related and that mutations in several of these genes affect germination in response to ABA and NaCl strengthens the argument that these interacting proteins could be involved in mediating signaling from these receptors to the ABA signaling pathway. One candidate from the yeast two-hybrid experiments is ABF2, which is part of the ABA signaling pathway. Yeast two-hybrid analysis showed that it potentially physically interacts with ETR2. If true, this provides one pathway by which ETR2 levels might affect ABA signaling. However, this does not explain how ETR1 is affecting ABA signaling. An additional problem with this simple model is that loss of ABF2 alone, or in combination with loss of ABF3 and ABF4, has no measurable effect on seed germination in response to ABA (Fujita et al., 2005; Yoshida et al., 2010), indicating that ETR1 and ETR2 are having a more widespread effect to alter ABA signaling. Our observation that the transcript abundance of genes encoding for many ABA signaling components is affected by ETR1 and ETR2 supports this. If true, it will make it more difficult to determine a specific pathway between these ethylene receptors and ABA signaling. This is especially true because our data also suggest that ETR1 and ETR2 are indirectly affecting ABA signaling.

Cross talk between ethylene signaling and other hormone signaling pathways has been described and usually involves signaling from downstream components such as EIN2 or EIN3 as hub points between the ethylene pathway and other hormone signaling pathways (Muday et al., 2012; van de Poel et al., 2015). Of particular note, in Arabidopsis, CTR1 and EIN2 mutants affect responses to ABA, indicating cross talk from these core components of ethylene signaling to the ABA signaling pathway (Beaudoin et al., 2000; Ghassemian et al., 2000; Wang et al., 2007; Subbiah and Reddy, 2010; Thole et al., 2014). In *Physcomitrella patens*, CTR1 modulates both ethylene and ABA signal transduction, suggesting an ancestral role of CTR1in both pathways (Yasumura et al., 2015). However, in this study we describe cross talk from the ethylene receptors to affect ABA signaling independently of the canonical ethylene signal transduction pathway. Additionally, we show that ABA affects ethylene receptor transcription and this transcriptional regulation is affected by the ethylene receptors. Even though the exact pathway for these complex interactions still needs to be resolved, the fact that this cross talk originates at receptors for one hormone to affect signaling for another hormone is intriguing, and suggests that there are likely to be other instances where receptors are signaling to other pathways via noncanonical pathways.

MATERIALS AND METHODS

Plant Materials and Chemicals

The Arabidopsis (*Arabidopsis thaliana*) mutants used are in the Columbia background, which was used as the wild-type control. The *etr1-6*, *etr2-3*, and *etr1-6;etr2-3;ein4-4* triple mutants are lab stocks originally described by Hua and Meyerowitz (1998). The triple mutant plants transformed with *cDNA* encoding the full-length *ETR1* (*cETR1*) or truncated *ETR1* lacking the receiver domain (*cetr1-ΔR*), full-length *gETR1*, a full-length genomic transgene with a *D659A* or *E666A* point mutation, have previously been described (Wang et al., 2003; Binder et al., 2004a, 2004b, 2006; Kim et al., 2011; Bakshi et al., 2015). The plant hormone, ABA, was obtained from ACROS Organics and the ABA biosynthesis inhibitor, NF, was from Fluka.

The *ctr1-2* mutant plants are lab stocks originally described by Kieber et al. (1993). The *etr1-6;ctr1-2*, and *etr2-3;ctr1-2* double-mutant plants were generated by crossing the appropriate parents. The resultant crosses were allowed to self-pollinate and taken to the F4 generation. In each generation, seedlings were grown in the dark for 3 d in the presence or absence of 1 μL L^{-1} ethylene to identify *ctr1-2*-containing plants. The *ctr1-2*-containing crosses were identified

by the constitutive ethylene response phenotype where seedlings grew slowly and with an exaggerated apical hook when grown in ethylene-free air in the dark. The plants were then genotyped for *etr1-6* or *etr2-3*. To distinguish *ETR1* from *etr1-6*, we used the primers and methods of Kim et al. (2011), where *etr1-6* gives a larger product than *ETR1*. For *ETR2* and *etr2-3*, we used the ETR2-1w, ETR2-1m, and ETR2-41 primers and methods described by Hua and Meyerowitz (1998). A product formed by the ETR2-1m and ETR2-4 primer pair indicated the presence of *etr2-3*, whereas a product formed with the ETR2-1w and ETR2-41 primer pair indicated the presence of the wild-type *ETR2*.

The *pyk10-1*, *leb-1*, SALK 065256, SALK 090550, SALK 119971, and SALK 063827 mutants were obtained from the Arabidopsis Biological Resource Center. The *pyk10-1* and *leb-1* mutants have previously been described (Nagano et al., 2008, 2009). To confirm that the four SALK lines were homozygous T-DNA insertional mutants, we ran PCR reactions with gene-specific primer pairs for each SALK line. The primers used for genotyping were: SALK 065256 5′-CTGAAGAAGAAATGGGGTTCC-3′ (forward), SALK 065256 5′-GTTTATTCCCTCCACTGCTCC-3′ (reverse), SALK 090550 5′-ATACACGAAACCCAACAGCTG-3′ (forward), SALK 090550 5′-GAAAGCAGTTGCTCATCAACC-3′ (reverse), SALK 119971 5′-ATTGGTACTATGTGCGGGTTG-3′ (forward), SALK 119971 5′-GGAAGAGAGGAGGCACAAATC-3′ (reverse), SALK 063827 5′-GTTTCTCACCAGATCGGGATC-3′ (forward), and SALK 063827 5′-GCAATACCAAAAGCAAGATGC-3′ (reverse).

We also confirmed the presence of the insertion by using the reverse primer for each SALK line paired with the LBb1.3 left border primer (5′-ATTTTGCCGATTTCGGAAC-3′) designed by the Salk Institute Genomic Analysis Laboratory for the T-DNA insertion. PCR cycling was performed at 95°C for 4 min for one cycle, followed by 40 cycles consisting of an initial denaturation at 95°C for 40 s, annealing at 52°C for 40 s, and extension at 72°C for 3 min. The final cycle was followed by a 5-min extension phase at 72°C. The lack of a product with the gene-specific primer pair and the presence of a product when using the left border-reverse primer pair indicated the plants were homozygous mutants for the gene in question.

Seed Germination Experiments

Seed germination studies were conducted using the methods of Wilson et al. (2014a, 2014b). To reduce biological variation, plants were grown at the same time under uniform conditions with seeds collected on the same day, then stored in a desiccator. After at least 3 weeks, seeds were mechanically sorted so that we used seeds between 250 and 300 μm in size (Hensel et al., 1993; Elwell et al., 2011). We surface-sterilized the seeds for 30 s with 70% (v/v) ethanol, then allowed the seeds to dry and placed them on 0.8% (w/v) agar plates that contained half-strength Murashige and Skoog salt (Murashige and Skoog, 1962), pH 5.7 fortified with vitamins and no added sugar. At least three plates with 20 seeds each were plated for each seed line and condition as indicated. Plates were wrapped with porous surgical tape to avoid accumulation of ethylene (Buer et al., 2003). Unless otherwise specified, plates were kept at 20°C to 21°C under 12 to 13 μmol m^{-2} s^{-1} white light with a long-day photoperiod (16 h light/8 h dark). Seed germination was evaluated every 12 h and considered complete with the rupture of the testa (seed coat). For experiments with ABA and NF, control plates contained 0.01% (v/v) ethanol as a solvent control. For experiments where seed germination was carried out in darkness, seeds were placed on agar plates under dim light, then treated with far-red illumination for 5 min as previously described (Oh et al., 2007; Wilson et al., 2014a, 2014b), and kept in darkness for 7 d at which time the percent of seed germination was determined.

Plasmid Construction

Full-length coding sequences of *RD21A*, *LEB*, and *DI19* and C-terminal coding sequences of *ETR1* and *ETR2* were isolated from cDNA of wild-type Columbia seedlings. The coding sequences of these genes were PCR-amplified using forward and reverse primers containing specific restriction enzyme sites (Supplemental Table S2). PCR amplification was performed using New England Taq DNA polymerase following the manufacturer's instructions. PCR products of the cytosolic domains of *ETR1* or *ETR2* were restriction-enzyme-digested using *Eco*RI and *Bam*HI restriction enzymes, purified and fused to the GAL4 DNA binding domain of pGBKT7 vector (Clontech) to generate pGBKT7-ETR1 and pGBKT7-ETR2. Similarly, PCR products of the full-length coding sequences of the interacting proteins were double-restriction-enzyme digested, purified, and fused to the GAL4 DNA activation domain of pGADT7 vector (Clontech) to generate pGADT7-RD21A, pGADT7-LEB, and pGADT7-DI19. All the constructs were verified by double-restriction-enzyme digestion giving the correct size products and by sequencing.

Yeast Two-Hybrid Screens

To conduct yeast two-hybrid screens, bait constructs encoding for the cytosolic portion of ETR1 or ETR2 fused to the GAL4 DNA binding domain of pGBKT7 vector were introduced into yeast (*Saccharomyces cerevisiae*) strain Y187 to generate the bait strain. These were used to screen cDNA prey libraries generated in yeast strain AH109 as a fusion to the GAL4 activation domain as described in Hewezi et al. (2008). Screening for interacting protein partners and subsequent analyses were performed as described in BD Matchmaker Library Screening Kits (Clontech). Selected interactions identified in the yeast two-hybrid screens were first tested using yeast cotransformation assays. For the cotransformation assay, *S. cerevisiae* strain AH109 cells were cotransformed with pGBKT7-ETR1 or pGBKT7-ETR2 and pGADT7-RD21A, pGADT7-LEB, and pGADT7-DI19 constructs and interactions were assayed using a stringent selection on synthetic quadruple drop-out media SD/-Ade/-His/-Leu/-Trp selective medium performed in triplicate. Control growth conditions were carried out on double drop-out media (SD/-Leu/-Trp). Serial dilutions of yeast cotransformed cells were used to measure the strength of the interactions.

Gene Coexpression Network Analysis

To determine whether an interaction partner was coexpressed with ETR1 or ETR2, we used methods modified from Piya et al. (2014). Briefly, the coexpression profiles of *ETR1*, *ETR2*, and target genes were analyzed from different Arabidopsis tissues and organs including embryos, floral buds, flowers, hypocotyls, leaves, roots, shoot apical meristem, seedlings, and whole plants using 63 different RNAseq datasets from the Sequence Read Archive (Leinonen et al., 2011). The individual gene expression level from all the RNAseq data sets was quantified and represented as values measured in fragments per kilobase of transcript per million mapped reads (FPKMs). FPKM value of 1.0 was set as the threshold for expressed genes and hence, only those genes having FPKM values greater than 1 in at least one tissue were included in the gene coexpression analysis. To identify the tissues where the interacting pairs were coexpressing, we calculated the Z-scores for each of the FPKM values. The Z-score values were averaged across different samples of a given tissue and positive Z-score values with $P < 0.05$ represent high expression. Next, the sample contribution scores were calculated by multiplying Z-scores of ETR1 or ETR2 with the interacting partners for each tissue as described in Obayashi et al. (2014) and then the values were used to construct the heatmap using the Multiple Experiment Viewer. Red color represented pairs with highly correlated gene coexpression profiles and green represented highly anticorrelated pairs. Positive sample contribution score values obtained from multiplying two negative Z-scores were considered negative. Based on this information, we generated a gene coexpression network using Cytoscape (Shannon et al., 2003). The network contained 83 nodes and 87 edges representing the interacting combinations. Gene coexpression profiles of ETR1, ETR2, and target genes were analyzed in Arabidopsis root tissues using 10 different RNAseq datasets from the SRA. Pairwise coexpression values of genes encoding ETR1, ETR2, and target proteins in root tissues were used to generate the coexpression network of the ETR1, ETR2, and the target interacting proteins. Continuous edges indicated significant coexpression in root, dashed edges represented significant coexpression with the receptor in at least one tissue other than root, and red dotted edges indicated no significant coexpression in any tissue with their respective receptor partner.

BiFC

To determine whether selected proteins interacted with ETR1 or ETR2 in planta, we used BiFC assays as described by Hewezi et al. (2008). The full-length coding sequence of *ETR1* or *ETR2* were PCR-amplified from cDNAs of wild-type Columbia seedlings using forward and reverse primers containing specific restriction enzyme sites (Supplemental Table S2). After double-restriction digestion of the constructs, the PCR products were cloned into pSAT4-nEYFP-C1 to generate either N terminus-tagged ETR1 and ETR1 (YN-*ETR1* and YN-*ETR2*, respectively) or C terminus-tagged receptors (*ETR1*-YN and *ETR2*-YN) fusions using the specific restriction enzymes. Similarly, the

full-length coding sequences of *RD21A*, *LEB*, and *DI19* were cloned into pSAT4-cEYFP-C1B to generate YC-*RD21A*, YC-*LEB*, and *DI19*-YC fusions. All these generated constructs were confirmed by double-restriction-enzyme digestion and sequencing. All combinations of nEYFP and cEYFP fusions, in addition to negative and positive controls, were coexpressed in *Nicotiana benthamiana* leaves using particle bombardment as previously described by Crawford and Zambryski (2000). All the bombardments were performed in triplicates in two independent experiments. Cotransformed leaf tissues were incubated at 25°C in dark for 16 h to 24 h before being detected for YFP signal. Bright and fluorescent images were captured using a Leica SP8 White Light Laser Confocal System.

RNA Isolation and Real-Time qRT-PCR

To evaluate the transcript abundance of Arabidopsis genes, we used real-time qRT-PCR. To do this, total RNA was isolated from 25 mg (dry weight) of seeds that were germinated in the presence or absence of 1 μM ABA or 150 mM NaCl for 2 d. We used the methods of Meng and Feldman (2010) as modified by Wilson et al. (2014a, 2014b) to isolate RNA. Transcript data were normalized to At3g12210 (Dekkers et al., 2012) using the method of Livak and Schmittgen (2001) for each seed line for each condition to obtain the relative amounts of target gene transcripts between plant backgrounds for each treatment. These levels were then normalized to the levels observed in untreated wild-type seeds. The primer pairs used for qRT-PCR of the receptors were: 5′-AGTGTTAAGACTCGGGAGCTT-3′ (forward) and 5′-GTTTCTTCCTGAGTTC-GAATCAAT-3′ (reverse) for *ETR1*, 5′-ATGGCGTTTACTGTTTTCAAGATG-3′ (forward) and 5′-CAAAATCAAACCAACTTCACGACC-3′ (reverse) for *ETR2*, 5′-ACTCATTTGCTTAACGCTTGGACGTAT-3′ (forward), and 5′-AAGCTCTCTAACTTTCCATTTCAACAG-3′ (reverse) for *EIN4*, 5′-ATTGCTAAAGTCTCTTGCGCGGTTGTG-3′ (forward) and 5′-TCTCTATC-TAACTCATCAGCTTTCTTC-3′ (reverse) for *ERS1*, and 5′-TCACTGGCCTTGGGT-CATGACAGCTGT-3′ (forward) and 5′-CTCTGGTCTTCTTACTCAACATAAACT-3′ (reverse) for *ERS2*. All other primers used for this analysis have been described previously (Fujii et al., 2007; Miura et al., 2009; Bhaskara et al., 2012; Chan, 2012; Gliwicka et al., 2012; Singh et al., 2015; Yoshida et al., 2015; Zhou et al., 2015; Zhang et al., 2016).

Accession Numbers

Arabidopsis Genome Initiative accession numbers for genes studied in this article are *ABF1*, At1g49720; *ABF2*, At1g45249; *ABF3*, At4g34000; *ABF4*, At3g19290; *ABI1*, At4g26080; *ABI2*, At5g57050; *ABI5*, At2g36270; *AHG1*, At5g51760; *AHG3*, At3g11410; *CRA1*, At5g44120; *CTR1*, At5g03730; *DI19*, At1g56280; *EIN4*, At3g04580; ERS1, At2g40940; ERS2, At1g04310; *ETR1*, At1g66340; *ETR2*, At3g23150; *HAB1*, At1g72770; *HAB2*, At1g17550; *HAI1*, At5g59220 ; *HAI2*, At1g07430; *HAI3*, At2g29380; *KIN1*, At5g15960; *LEB1*, At3g09260; *PYL1*, At5g46790; *PYL5*, At5g05449; *PYL7*, At4g01026; *PYL9*, At1g01360; *PYR1*, At4g17870; *RAB18*, At5g66400; *RD21A*, At1g47128; *RD29A*, At5g52310; *SLAC1*, At1g12480; *SLAH3*, At5g24030; *SnRK2.2*, At3g50500; *SnRK2.3*, At5g66880; *SnRK2.6*, At4g33950; *SnRK2.10*, At1g60940.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Seed germination time-courses under different inhibitory conditions.

Supplemental Figure S2. The effect of NF on seed germination under different inhibitory conditions.

Supplemental Figure S3. Seed germination time-courses in various inhibitory conditions for ETR1 receiver domain mutants.

Supplemental Figure S4. Seed germination time-courses in the dark for ETR1 receiver domain mutants.

Supplemental Figure S5. Genotyping and phenotyping of double mutant plants containing *ctr1-2*.

Supplemental Figure S6. Time for 50% seed germination of receptor mutant plants crossed with *ctr1-2* in response to ABA.

Supplemental Figure S7. Seed germination time-courses of mutant seed lines in response to ABA.

Supplemental Figure S8. Simple working genetic model for relationships among ETR1, ETR2, and interacting proteins in the control of seed germination.

Supplemental Table S1. List of proteins that interact with the cytosolic portion of ETR1 or ETR2 or both in the yeast two-hybrid screen.

Supplemental Table S2. List of primers used for cotransformation and BiFC analyses.

ACKNOWLEDGMENTS

The authors acknowledge the technical help of Andrew Ward, Joanna Nosarzewska, and NaSha Austin, and the helpful advice of Rebecca Wilson, Tessa Burch-Smith, Eric Schaller, and Anna Stepanova.

Received October 3, 2017; accepted November 17, 2017; published November 20, 2017.

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