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Arabidopsis CPR5 regulates ethylene signaling via molecular association with the ETR1 receptor

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Abstract

The plant hormone ethylene plays various functions in plant growth, development and response to environmental stress. Ethylene is perceived by membrane-bound ethylene receptors, and among the homologous receptors in *Arabidopsis*, the ETR1 ethylene receptor plays a major role. The present study provides evidence demonstrating that *Arabidopsis* CPR5 functions as a novel ETR1 receptor-interacting protein in regulating ethylene response and signaling. Yeast split ubiquitin assays and bi-fluorescence complementation studies in plant cells indicated that CPR5 directly interacts with the ETR1 receptor. Genetic analyses indicated that mutant alleles of *cpr5* can suppress ethylene insensitivity in both *etr1-1* and *etr1-2*, but not in other dominant ethylene receptor mutants. Overexpression of *Arabidopsis CPR5* either in transgenic *Arabidopsis* plants, or ectopically in tobacco, significantly enhanced ethylene sensitivity. These findings indicate that CPR5 plays a critical role in regulating ethylene signaling. CPR5 is localized to endomembrane structures and the nucleus, and is involved in various regulatory pathways, including pathogenesis, leaf senescence, and spontaneous cell death. This study provides evidence for a novel regulatory function played by CPR5 in the ethylene receptor signaling pathway in *Arabidopsis*.

INTRODUCTION

The gaseous phytohormone ethylene plays an important role in various plant processes, including seed germination, promotion of fruit ripening, organ senescence, abscission, apical hook formation, flowering and gravitropism (Abeles et al. 1992). Ethylene treatment can result in inhibition of dark-grown *Arabidopsis* seedling root and hypocotyl elongation, hypocotyl radial swelling, and an exaggerated apical hook (Bleecker et al. 1988; Guzman and Ecker 1990), termed the “triple response”. By screening for mutants exhibiting an altered “triple response” phenotype, many core components in the ethylene signal pathway

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AUTHOR CONTRIBUTIONS

F.W., L.W. and L.Q. performed most of the research and F.W. wrote the first draft of the manuscript. J.C. performed some protein interaction experiment and M.B.P. carried out the mutant screenings. H.P. and T.Z. revised the manuscript. C.C. and C.H.D. designed the experiments and revised the manuscript.

have been isolated, including ethylene receptors, a MAPKKK protein CTR1, the endoplasmic reticulum (ER) membrane-localized protein, EIN2, and the transcription factor EIN3/EIL (Chang et al. 1993; Hua et al. 1995, 1998; Chao et al. 1997; Sakai et al. 1998; Alonso et al. 1999). Ethylene signaling starts with ethylene binding to the ER membrane-associated receptors, and finally leads to changes in gene expression within the nucleus (Guo and Ecker 2004).

In *Arabidopsis thaliana*, there are five homologous ethylene receptors (ETR1, ERS1, EIN4, ETR2, ERS2), which resemble the two-component histidine protein kinase family primarily found in prokaryotes (Chang et al. 1993; Hua et al. 1995, 1998; Sakai et al. 1998). These receptors are negative regulators of ethylene responses (Hua and Meyerowitz 1998; Hall and Bleecker 2003; Qu et al. 2007) that, upon binding ethylene with the help of a copper cofactor are inactivated, leading to triggering of the ethylene response; however, in the absence of ethylene, the active receptors block ethylene signaling (Rodriguez et al. 1999).

According to their structure similarities, these receptors can be divided into two subfamilies: subfamily I contains ETR1 and ERS1, and subfamily II is comprised of ETR2, ERS2 and EIN4. The subfamily I receptors have three N-terminal transmembrane domains, and a highly conserved histidine kinase domain, whereas the subfamily II receptors have four N-terminal transmembrane domains and a degenerated histidine kinase domain, and they may have Ser/Thr kinase activity (Gamble et al. 1998; Moussatche and Klee 2004; Chen et al. 2009). All five ethylene receptors contribute to ethylene signaling and are redundant in regulating ethylene responses, while subfamily I receptors, and especially ETR1, play a predominant role (Hua et al. 1995; Hua and Meyerowitz 1998; Hall and Bleecker 2003; Qu et al. 2007; Liu et al. 2010).

RTE1 (*REVERSION TO ETHYLENE SENSITIVITY* 1) was identified as a positive and upstream regulator of the *ETR1* receptor, and this regulation is specific genetically (Resnick et al. 2006; Resnick et al. 2008; Rivarola et al. 2009). RTE1 is an activator of ETR1, which was also supported by the direct interaction of the two proteins and their co-localization (Dong et al. 2008, 2010). Conserved RTE1 homologs are widely distributed in higher eukaryotes (Resnick et al. 2006). *Arabidopsis RTE1* encodes an integral membrane protein with three homologs in tomato, two of which were shown to regulate ethylene responses in this crop plant (Barry and Giovannoni 2006; Klee 2006; Ma et al. 2012). In *Rosa hybrida*, the expression of *Rh-RTH1* was responsive to ethylene, and was also partially correlated with expression of *Rh-ETR1* and *Rh-ETR3* (Yu et al. 2010). In addition, it was reported that the rice *RTE1* homolog (*OsRTH1*, Zhang et al. 2012) and the *RTE*-like genes (*DCRTE1* and *DCRTH1*) of carnation (Yu et al. 2011) participate in the regulation of ethylene responses in seedling growth and flower senescence.

Arabidopsis RTE1 is localized to ER and Golgi membranes (Resnick et al. 2006; Zhou et al. 2007; Dong et al. 2008). Furthermore, based on a yeast two-hybrid screen, it was shown that the ER-localized cytochrome B5 (cb5) and lipid transfer protein LTP1 could directly interact with RTE1 and function in modulation of ethylene responses and signaling (Chang et al. 2014; Wang et al. 2016). The *Arabidopsis* Cb5 proteins are likely involved in electron transfer reactions, as in all eukaryotes. Both *Cb5-D* and *LTP1* might act upstream of *RTE1*

in regulating the ETR1-mediated repression of ethylene responses. Both *atcb5* and *atltp1* mutants show increased ethylene sensitivity, whereas overexpression of *Cb5-D* or *LTP1* conferred decreased ethylene sensitivity. It appears that both AtCb5 and AtLTP1 play positive roles in ethylene signaling and responses, probably by participating in the protein complex involved in regulation of the ethylene signal, via RTE1. These findings suggest that ETR1 receptor signaling might be tightly controlled by a protein complex containing RTE1 and the RTE1-binding proteins. It is conceivable that other unknown regulators might exist in the ETR1 receptor signaling pathway.

The *Arabidopsis CPR5* (*CONSTITUTIVE EXPRESSOR OF PATHOGENESIS-RELATED GENES 5*) gene was isolated based on a screen for constitutive expressions of systemic acquired resistance (SAR) (Bowling et al. 1997; Boch et al. 1998). Recently, it was reported that the *Arabidopsis* CPR5 (hereafter CPR5) acts as a nucleoporin in controlling effector-triggered immunity (ETI) and programmed cell death (PCD) in plants (Gu et al. 2016). During plant growth and development, *CPR5* regulates endoreduplication, cell division, cell expansion and spontaneous cell death (Kirik et al. 2001; Brininstool et al. 2008; Perazza et al. 2011). It was proposed that CPR5 participates in a complex interacting with cell cycle regulators, and plays a critical role in this process (Bao and Hua 2014). *CPR5* also participates in plant stress responses, including thermotolerance (Wang et al. 2012), ABA signaling (Gao et al. 2011), cellular ROS status and/or signaling (Jing and Dijkwel 2008; Jing et al. 2008), and K^+ homeostasis (Borghi et al. 2011). As a positive modulator, CPR5 regulates plant growth under physiological conditions and during stress by antagonizing SA-dependent growth inhibition through the unfolded protein response (UPR) pathway (Meng et al. 2017).

A role for CPR5 in ethylene signaling was suggested by earlier studies in which it was reported that exogenous application of the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), to *Arabidopsis cpr5/hys1/old1* mutant seedlings gave an enhanced ethylene response (Jing et al. 2005, 2007; Aki et al. 2007). However, little is currently known concerning the underlying molecular mechanism by which CPR5 might act in the ethylene signaling pathway. In the present study, we provide data showing that AtCPR5 plays a critical role in the regulation of ethylene signaling, via direct association with the ETR1 receptor.

RESULTS

Screening for suppressors of *etr1-2* ethylene insensitivity

Previously, a genetic screen for suppressors of ethylene insensitivity performed on the *etr1-2* mutant identified a positive regulator of the ETR1 receptor (Resnick et al. 2006). In order to identify additional potential ETR1 regulators, a genetic screen was conducted using a new pool of ethyl methyl sulfonate (EMS)-mutagenized *etr1-2* plants. To this end, dark-grown seedlings were treated with ethylene and screened for *etr1-2* suppressor mutants that exhibited the “triple-response” phenotype. A mutant displaying this restored ethylene “triple-response” was chosen for in-depth analysis. To assess whether the mutation was intragenic or extragenic to the *etr1-2* allele, this mutant was crossed to wild type (Col-0) and

the resultant F_1 progeny was analyzed for the etiolated seedling "triple response" phenotype on MS agar plates containing ACC (100 μM).

Due to the dominant nature of *etr1-2*, the resulting progeny of the F_1 seedlings displayed an ethylene-insensitive phenotype, suggesting that the mutation was extragenic to *etr1-2*. We next twice backcrossed this mutant line to *etr1-2* to remove extraneous mutations. A mapping population was made by crossing the mutant with a dominant ethylene insensitive *etr1* allele, in the *Arabidopsis* ecotype *Landsberg erect* (Resnick et al. 2006). Using simple sequence length polymorphism (SSLP) markers, we mapped the extragenic mutation locus to a 68-kb interval on the bottom of chromosome 5. After sequencing the corresponding DNA fragments of 9 genes within this region, we identified a single nucleotide C-to-T missense mutation which codes for Pro535 in place of Ser535 in the open reading frame (ORF) of gene AT5G64930. This is the same mutation as in *hys1-2* (Yoshida et al. 2002), an allele of *CPR5* (Bowling et al. 1997). The etiolated mutant seedling is shown in Figure 1A (see *etr1-2 hys1-2*). The other genes within this region were sequenced but no further sequence differences were detected.

We next tested a second allele by obtaining the *cpr5-1* mutant described in Bowling et al. (1997), crossing it with *etr1-2*, and identifying the double mutant *etr1-2 cpr5-1* in the F_2 generation. Ethylene "triple response" analysis established the same suppression of ethylene insensitivity in these *etr1-2 cpr5-1* plants (Figure 1A, B), providing further support that the *cpr5* mutations could affect ETR1-mediated ethylene signaling.

Disruption of *CPR5* significantly affects ethylene signaling

We next analyzed a *cpr5* knockout allele (*cpr5-T3*) using a T-DNA insertion line from the Arabidopsis Biological Resource Center (ABRC, Ohio State University, Columbus). Quantitative RT-PCR revealed that *CPR5* transcripts were barely detected in this T-DNA insertion mutant (Figure 1C). To examine the ethylene sensitivity of the *cpr5-T3* mutant, gaseous ethylene was applied to these plants. Our assays indicated that *cpr5-T3* had more yellow leaves compared to wild type following ethylene treatment for 3 days (Figure 1D). Here, *etr1-2* plants were used as a control and they were always ethylene insensitive. Interestingly, senescent leaves were observed in the sealed box test system after 3 days, even without injection of exogenous ethylene, raising the possibility that *cpr5-T3* plants could have enhanced ethylene sensitivity. Assays using dark-grown etiolated seedlings treated with ACC, at different concentrations, confirmed that *cpr5-T3* seedlings had an enhanced ethylene response (Figure 1E). In the absence of ethylene treatment, *cpr5-T3* seedlings failed to display a hypersensitivity response (Figure 1E).

To test whether *cpr5-T3* could suppress the *etr1-2* ethylene insensitivity, a genetic cross of *cpr5-T3* with *etr1-2* was performed and the double mutant, *etr1-2 cpr5-T3*, was identified from the F_2 generation. As expected, the *cpr5-T3* mutation could suppress the ethylene insensitivity of *etr1-2* (Figure 1A, B). Taken together, these results support the notion that *CPR5* plays a regulatory function in the ethylene response.

CPR5 overexpression enhances the ethylene response and signaling

To advance our understanding of the role played by *CPR5* in regulating ethylene responses, an approach of over-expressing *CPR5* in transgenic *Arabidopsis* was employed. An *Arabidopsis CPR5* full length cDNA was amplified by PCR and the resultant product was cloned under the strong *CaMV35S* promoter. A binary vector harboring *p35S::CPR5* was transferred into *Agrobacterium* for flower dip transformation of *Arabidopsis thaliana* plants (Clough and Bent 1998). Seven transgenic lines were examined for *CPR5* transcript levels by RT-PCR; all transgenic lines showed high levels of *CPR5* transcripts (Figure 2A). Under treatment with different concentrations of ACC (0, 0.5, 20 μM), the etiolated seedlings of *CPR5*-overexpressing (*OX*) lines showed a significant difference compared to wild type plants, and all *OX* lines exhibited shorter hypocotyls (Figure 2B, C), indicating an increase in *CPR5* transcripts conferred enhanced ethylene sensitivity.

The *p35S::CPR5* construct was also introduced into tobacco (*Nicotiana tabacum*) mediated by *Agrobacterium*-mediated transformation (Supplement S2). The *CPR5* transcript levels were assayed by RT-PCR in three transgenic lines; all showed high levels (Figure 2D). The ethylene response of dark-grown wild type and *CPR5*-overexpression (*OX*) transgenic tobacco 6-day-old seedlings were analyzed in response to a range of ACC concentrations (0, 0.5, 5, 20, 100 μM) (Figure 2F) Interestingly, the hypocotyl length of *CPR5-OX* seedlings was significantly shorter than those of wild type seedlings, especially at low ACC concentrations (Figure 2E, F). This finding provides support for the notion that ectopic expression of *Arabidopsis CPR5* can enhance ethylene sensitivity in tobacco plants.

Altered expression of downstream *ERFs* in *cpr5-T3* and *CPR5-OX* plants supports a regulatory function of *CPR5* in ethylene signaling

To examine the effect of *CPR5-OX* on the endogenous transcript levels of ethylene responsive factors (*ERFs*) in the *Arabidopsis CPR5-OX* transgenic plants, nine genes (*ERF1*, *2*, *4*, *5*, *8*, *9*, *11*, *104*, and *105*) were chosen for quantitative RT-PCR analysis. These ethylene-induced *ERFs* were chosen based on information from The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org/) and Wang et al. (2016). Our assays indicated that the transcript levels for almost all the examined *ERFs* in the *CPR5-OX* (#*OX3* and #*OX7*) were higher than those in the wild type, indicating that increased *CPR5* transcript levels promote expression of downstream *ERFs* in these plants (Figure 3A).

In contrast to the elevated *ERF* transcript levels in the *CPR5-OX* transgenic plants, *ERF* expression in *cpr5* plants was lower than in wild type plants, among all the examined *ERFs* when no exogenous ACC was applied (Figure 3B–G). Surprisingly, we found that in *cpr5-T3* plants transcript levels for these *ERFs* were higher than those in the wild type plants, when exogenous ACC was applied (Figure 3B–G). These findings offer a clue as to why *cpr5* plants exhibited enhanced ethylene sensitivity (Figure 1D, E) following application of exogenous ethylene or its precursor (ACC). In addition, we noted that exogenous application of ACC could only slightly promote *ERF* expression (*ERF1* and *ERF9*) in *CPR5-OX* plants, implying that high levels of endogenous *ERFs* in these transgenic lines might restrain further induction of *ERFs* by exogenous ethylene.

Genetic analyses indicate that CPR5 mainly affects the ETR1 receptor

In order to examine whether the dominant ethylene-insensitive mutations in other ethylene receptor and downstream genes are suppressed by *cpr5*, genetic crosses of the ethylene insensitive mutants with *cpr5-T3* were performed and the following double mutants were obtained; *cpr5-T3 etr2-1*, *cpr5-T3 ers1-10* and *cpr5-T3 ein2-1*. Seedling "triple response" phenotypic analyses showed that *cpr5-T3* could not suppress the ethylene insensitivity of *etr2-1*, *ers1-10* or *ein2-1* (Figure 4B, C). These findings suggested that *CPR5* might regulate mainly ETR1 receptor signaling.

We also tested whether *CPR5* disruption could suppress another ETR1 mutant allele, *etr1-1*, a gain-of-function allele conferring ethylene insensitivity. A genetic cross of *cpr5-T3* with *etr1-1* was performed and the double mutant, *etr1-1 cpr5-T3* was identified from an F_2 population using molecular markers. Seedling "triple response" phenotypic analysis clearly confirmed that *cpr5* could suppress the ethylene insensitivity of *etr1-1* (Figure 4A). This result is different from that of *rte1*, which suppresses the ethylene insensitivity of *etr1-2* but not that of *etr1-1* (Resnick et al. 2006), indicating that CPR5 functions in ethylene signaling in a manner different from that of RTE1.

Molecular association of CPR5 with the ETR1 ethylene receptor

The fact that *CPR5* functions in regulating ethylene signaling prompted us to examine whether CPR5 could directly interact with the ETR1 receptor. At first, we performed a yeast split-ubiquitin assays (Chang et al. 2014; Wang et al. 2016). In frame fusions of Cub-ETR1 and Nub-CPR5 constructs were verified by DNA sequencing, and protein interactions were examined on selective medium lacking adenine, histidine, leucine and tryptophan. Strong protein interactions were detected in yeast cells co-expressing Cub-ETR1 and Nub-CPR5, whereas no colonies survived on the selection medium if either construct was absent (Figure 5A). It is interesting to note that the protein interaction between CPR5 and RTE1 was not detected, while a positive interaction between CPR5 and LTP1 was observed.

We next examined protein interaction between CPR5 and ETR1, *in planta*, using the bimolecular fluorescence complementation (BiFC) assay. The coding sequences of the YFP halves, *cYFP* and *nYFP*, were fused to the full-length coding sequences of *CPR5* at the N-terminus and *ETR1* at the C-terminus, respectively. In- frame fusion of the resultant constructs was verified by DNA sequencing. When cYFP-CPR5 and ETR1-nYFP fusions were transiently co-expressed in onion epidermal peel cells, a fluorescent signal was readily detected (Figure 5B). In our controls, fluorescence was not detected in cells if the cYFP-CPR5 and RTE1-nYFP fusions were co-expressed. In contrast, strong fluorescence was observed when the nYFP-CPR5 and LTP1-cYFP fusions were co-expressed. These BiFC assays indicated that CPR5 can physically interact with the ETR1 receptor, *in vivo*.

DISCUSSION

CPR5 is a novel ETR1 interacting protein

CPR5 is a membrane protein with five transmembrane helices at the C terminus and is associated with the endomembrane system, including the nuclear envelope and ER-

associated large granules. It is known that in *Arabidopsis*, *CPR5* is involved in a number of regulatory pathways, including the SAR pathway (Bowling et al. 1997; Gu et al. 2016), *RPS2* signal transduction pathway (Boch et al. 1998), cellular ROS status and/or signaling (Jing and Dijkwel 2008; Jing et al. 2008), the unfolded protein response (UPR) pathway (Meng et al. 2017), and regulation of endoreduplication, cell division, cell expansion and spontaneous cell death (Kirik et al. 2001; Perazza et al. 2011). An earlier study reported that an ectopically expressed GFP-CPR5 fusion protein, driven by the strong *35S* promoter, was localized to the nuclei of tobacco cells (Perazza et al. 2011), suggesting that CPR5 could be proteolytically cleaved and transported into the nucleus by an unknown mechanism. In this study, we show that CPR5 can physically interact with the ethylene receptor ETR1 (Figure 5A, B), which is localized predominantly to the ER membrane (Chen et al. 2002; Dong 2008; Grefen et al. 2008), and we provide evidence demonstrating that CPR5 plays a regulatory function in ETR1 receptor signaling. These findings implicate a new role for this ER-associated CPR5 protein.

CPR5 plays a key role in regulating ethylene signaling

Previously, enhanced ethylene responses of the *Arabidopsis cpr5/hys1/old1* mutant seedlings were observed when exogenous ethylene precursor ACC was applied to the culture medium (Jing et al. 2005, 2007; Aki et al. 2007). In our study, various approaches, including *cpr5* point mutations or gene knockout and *CPR5-OX* transgenic plants were used to probe the regulatory functions of *CPR5* in ethylene response and signaling. These studies indicated that CPR5 plays a crucial role in ethylene response and signaling, based on the following: (1) the *cpr5*/*hys1-2* point mutations could restore the ethylene sensitivity of *etr1-2* in the double mutant (Figure 1A, B, E); (2) a T-DNA insertion in *CPR5* gave rise to the *cpr5-T3* mutant exhibiting hypersensitive to exogenous ACC (Figure 1D, E); and (3) transgenic plants over-expressing *CPR5* either in *Arabidopsis* or ectopically in tobacco displayed enhanced ethylene sensitivity in the presence of exogenous ACC (Figure 2B, C, E, F). To gain insight into the underlying regulation, we examined expression of the downstream *ERFs* in the ethylene signaling pathway using quantitative RT-PCR. These assays revealed that, in the *cpr5-T3* mutant, endogenous transcript levels of these downstream *ERFs* were lower than in wild type plants (Figure 3B–G), whereas the levels in the *CPR5-OX* transgenic lines were higher than in wild type (Figure 3A), suggesting that *CPR5* functions as a key regulator in controlling expression of downstream *ERFs* that function in the ethylene signaling pathway.

It is also interesting to note that the levels of most of *ERFs* examined in the *cpr5-T3* mutant were higher than in wild type plants when exogenous ACC was applied, although their endogenous levels were lower than in the wild type controls (Figure 3B–G). These findings provide insight into why the *cpr5* mutant exhibits ethylene hypersensitivity in response to exogenous ethylene or its precursor (ACC) (Figure 1D, E). Currently, it is not known how high expressions of these *ERFs* might be modulated in the *cpr5* mutant.

This study provides evidence showing the molecular interaction between CPR5 and the ETR1 receptor (Figure 5), indicating that CPR5 might directly regulate the ETR1 receptor signaling. As CPR5 could function as a nucleoporin (Gu et al. 2016), knockout of *CPR5*

might affect the nucleocytoplasmic transport of mRNAs and the ETR1 protein localization to ER. Assays by *CPR5* over-expressing either in transgenic *Arabidopsis* or tobacco plants confirm further the regulatory function of *CPR5* in ethylene signaling. It was observed that increase of the *CPR5* transcripts enhances the ethylene sensitivity (Figure 2B, C, E, F). It is suggested that the over-produced CPR5 might sequester the ETR1 receptor protein, a negative factor in ethylene signaling, leading to higher expression of the downstream *ERFs* (Figure 3A) and enhanced ethylene sensitivity. However, the other regulation might also exist, for example, increase of the CPR5 levels might interfere with the unknown ethylene signaling component(s).

CPR5 functions differently from RTE1 in regulating ETR1 receptor signaling

In the past, limited information has been gained as to the molecular regulation of the ethylene receptors. Defects in *RAN1* severely disrupt the formation of wild type ethylene receptors because of improper copper loading (Hirayama et al. 1999; Woeste and Kieber 2000). In *ran1*, the *etr1-1* receptor is stable in the absence of copper and is locked into the repressor signaling state. Compared to RAN1, RTE1 is an activator of the ethylene signaling receptor, ETR1 (Resnick et al. 2006). RTE1 co-localizes with ETR1 at the ER and Golgi apparatus and they physically interact (Dong et al. 2008, 2010). It was proposed that RTE1 affects the conformation of the ETR1 ethylene-binding domain and/or the equilibrium state of ETR1, resulting in the promotion or stabilization of the signaling state of ETR1 (Resnick et al. 2008). However, it is still unclear why the *rte1* mutant is capable of suppressing the *etr1-2* but not the *etr1-1* allele (Resnick et al. 2006). The *etr1-2* encodes an Ala^{102}-to-Thr substitution in the putative ethylene binding domain, whereas the *etr1-1* mutation fully blocks ethylene binding (Schaller and Bleecker 1995; Hall et al. 1999), because of a Cys^{65}-to-Tyr substitution in this mutant prevents association with the copper cofactor (Rodriguez et al. 1999). The *etr1-1* mutant confers strong ethylene insensitivity, indicating that the signaling domain is essentially locked into an active conformation. In an earlier study, genetic analyses suggested that CPR5 is independent of ETR1 in controlling of leaf senescence (Jing et al. 2002). However, effect of the *cpr5*/*old1* mutation on the ethylene sensitivity of the double mutant was not examined. In the present study, we show that *cpr5* mutations can restore the ethylene sensitivity in both *etr1-2* and *etr1-1*, indicating that CPR5 may be upstream of the ETR1 receptor in regulation of ethylene signaling. Based on a recent finding that CPR5 could act as a nucleoporin in controlling of the nucleocytoplasmic transport of mRNAs (Gu et al. 2016), it is likely that CPR5 might be required for *ETR1* mRNA transport out of the nucleus for protein translation, or transport of ETR1 protein to the ER.

Genetic analyses using double mutants of *cpr5-T3* with each of three other ethylene insensitive mutants (*ers1-10*, *etr2-1*, and *ein2-1*), showed that the ethylene insensitivity displayed by these mutants was not suppressed by the *cpr5-T3*, indicating that the ETR1 ethylene receptor might well be the main target of CPR5 in the ethylene signaling pathway.

MATERIALS AND METHODS

Mutant screening and map-based cloning

For mutagenesis, 200 mg of the *etr1-2* seeds were washed once in a solution of 0.1% Tween 20 (Sigma Aldrich) and then incubated in a solution of 0.3% ethylmethanesulfonate (EMS) (Sigma Aldrich) for 12 hours with gentle agitation. After washing twice with sterile water the seeds were sown onto soil in a growth chamber. Seeds were collected from these M_1 plants and plated onto 100 μM ACC plates for suppressor screening based on the seedlings "triple response" phenotype in response to ethylene. Potential suppressor mutants were identified and verified in the next generation. Confirmed mutants were crossed to wild type (Col-0) and to *etr1-2* in order to determine whether the mutations were intragenic or extragenic and dominant or recessive, respectively. The selected suppressor mutants were backcrossed to *etr1-2* twice to remove extraneous mutations.

For genetic mapping, the selected mutants were crossed with an *etr1* mutant in the Landsberg *erect* (L*er*) background and the F2 population was used for PCR-based mapping, as previously described (Resnick et al. 2006). Mutants were scored as homozygous for *etr1-2* on the basis of the etiolated seedling "triple response" phenotype. A total of 526 individuals were selected for use in the simple sequence polymorphism (SSLP) marker-based mapping. Initial mapping of the selected suppressor mutant linked the mutation to the marker MVP7, MXK3 and MNA5 on the bottom of chromosome 5. Genomic DNA corresponding to candidate genes was PCR-amplified from mutant and wild type plants and sequenced to identify the mutation.

Genetic crosses and mutant genotyping

The *Arabidopsis* mutant *cpr5-1* and the double mutant *ein2-1 cpr5-1* were kindly provided by Dr. Xinnian Dong from Duke University (Bowling et al. 1997). The *Arabidopsis* T-DNA insertion line of *CPR5* (salk_074631, assigned as *cpr5-T3*) was obtained from the Arabidopsis Biological Resource Center (ABRC, Ohio State University, Columbus). The *cpr5-T3* mutant has a T-DNA insertion at exon 3 according to the ABRC. The double mutants *cpr5-T3 etr2-1*, *cpr5-T3 ers1-10* and *cpr5-T3 etr1-1* were generated by genetic crosses, and the F2 progeny from these crosses were screened by specific PCR markers, as previously described (Resnick et al. 2006; Wang et al. 2016).

Plant growth, transformation and ethylene response assays

Wild-type plants of *Arabidopsis thaliana* (ecotype Columbia (Col-0), or Landsberg *erecta* (L*er*)) and tobacco (*Nicotiana tabacum* NC89) were grown in 1/2 MS (Murashige and Skoog) medium or soil in a controlled environment growth chamber at 21°C under 16 h light/8 h dark. Transgenic *Arabidopsis* plants were generated by the floral dip infiltration method (Clough and Bent 1998) mediated by *Agrobacterium tumefaciens* (strain GV3101). Transformed plants were selected by BASTA, and the homozygous transgenic lines from the T3 generation were used for gene expression analysis and phenotypic characterization.

For tobacco transformation, pieces of tobacco leaves were inoculated with Agrobacterium in liquid culture (MS + 1.0 mg/L 6-BA + 0.1 mg/L NAA) for 10 min with gentle shaking, and

then cultured in darkness for 3 d. After washing, leaf discs were transferred to selection medium (MS +1.0 mg/L 6-BA + 0.1 mg/L NAA + 250 mg/L cefotaxime + 30 mg/L hygromycin). After propagation for ~30 d, transformed shoots were obtained. Hygromycin-resistant shoot buds were allowed to grow to ~2 cm in height and then plantlets were transferred into rooting medium (1/2 MS + 350 mg/L cefotaxime) for ~3 weeks before being transferred to soil. Transgenic seeds were collected 4–5 months later.

Infiltration of *Agrobacterium tumefaciens* (strain GV3101) was carried out, as previously described (Dong et al. 2010; Wang et al. 2016). In brief, 50 mL of Agrobacterium culture, in LB broth supplemented with antibiotics (50 mg/L kanamycin, 50 mg/L rifampicin), was precipitated, washed, and re-suspended in a solution containing 10 mM MgCl, 10 mM MES and 100 μM acetosyringone. Fresh onion peels were used for agroinfiltration. Generally, 20 independent agroinfiltrations were performed for each transformation construct.

The ethylene response assay of *Arabidopsis* seedlings was as previously described (Wang et al. 2016). In brief, seeds were surface sterilized and then sowed on 1/2 MS medium containing ACC at different concentrations (0, 0.5, 5, 20, or 100μM). After treatment at 4°C for 3 d, plates were moved to a growth chamber for 8 h under white light, then wrapped with aluminum foil and placed in a growth chamber for the indicated periods. Image J 1.48u software (http://rsb.info.nih.gov/ij/) was used to measure hypocotyl lengths. Statistical analyses were performed by one-way ANOVA with a 95% or 99 % confidence for significant difference.

For leaf senescence assays, ethylene treatment of adult plants was carried out in airtight clear acrylic chambers into which either ethylene gas or air was injected (Resnick et al. 2006). Plants were photographed after the chambers were placed in a growth room at 21°C for 3 d under 24-h lighting.

Yeast split-ubiquitin assays

For yeast split-ubiquitin assays, the open reading frame cDNA fragments of *CPR5* and *ETR1* were each PCR-amplified from existing templates with primers (*CPR5-For* (SfiI): ATTGGCCATTACGGCCATGGAAGCCCTCCTCCTCCCT; CPR5-Rev (SfiI): ATTGGCCATTACGGCCTCAAGCATAGTCAGACCCAC; ETR1-For (SfiI)-2: ATTGGCCATTACGGCCGAAGTCTGCAATTGTATTGA; ETR1-Rev (SfiI)-2: ATTGGCCGCCTCGGCCGGCATGCCCTCGTACAGTACCCG), cloned into a bait vector pPR3-N and a prey vector pBT3-STE through the restriction sites SfiI, and the resultant constructs, CPR5-pPR3-N and ETR1-pBT3-STE, were obtained, respectively. The vector construction of LTP1 or RTE1 for the yeast split-ubiquitin assay was as previously described (Wang et al. 2016). The insert sequences were verified by DNA sequencing, and protein interactions were examined, as previously described (Chang et al. 2014).

Constructs for BiFC assays

To generate constructs encoding the gene fusions of *cYFP-CPR5* and *nYFP-CPR5*, the PCR fragment of *cYFP* or *nYFP* was first amplified, based on the template pSPYCE or pSPYNE vector (Walter et al. 2004) with primers (cYFP-For: GCTCTAGAGCGACAAGCAGAAGAACGGCATCA; cYFP-Rev:

CGGGGTACCCCGCTTGTACAGCTCGTCCATGCCGAG; nYFP-For: GCTCTAGAGCATGGTGAGCAAGGGCGAG; and nGFP-Rev: CGGGGTACCCCGGGCCATGATATAGACGTTGTG) and cloned into the binary vector, pCambia1300-221-HA through restriction sites XbaI and KpnI. The coding sequence for full length *CPR5* was PCR-amplified from an existing *CPR5* cDNA template, using primers (Kpn I-CPR5 For: CGGGGTACCCCGATGGAAGCCCTCCTCCTCCCTCC, and BamHI-CPR5 Rev: CGGGATCCCGTCAAGCATAGTCAGACCCACCAT), and then cloned into the same vector through the restriction sites KpnI and BamHI. The insert sequences and the in frame fusions of *cYFP-CPR5* and *nYFP-CPR5* were verified by DNA sequencing. The same vectors containing the gene fusions of *ETR1-nYFP*, *LTP1-cYFP*, and *nYFP-RTE1* were used, as previously described (Dong et al. 2010; Wang et al. 2016).

Fluorescence microscopy

Imaging of fluorescent proteins in onion epidermal cells was conducted using a laser scanning confocal microscope (Leica TCS SP5). The excitation wavelength for YFP was 488 nm, and the emission filter wavelengths were 505–530 nm for YFP. Pieces of fresh onion peels were directly mounted into water on a glass slide for visualization. For each experiment, at least 15 different samples were examined and experiments were repeated three times.

RNA isolation, semi-quantitative and quantitative PCR analyses

Total RNA was isolated from light-grown seedlings with TRIzol (Sigma), and the reverse transcription of RNA was performed using PrimeScript TM RT Enzyme Mix, according to the manufacturer's recommendations (Takara Bio Inc, Otsu, Japan). Primers employed for Rt-PCR and quantitative RT-PCR are listed in Table S1. Quantitative RT-PCR analysis was performed on an Agilent Real-Time qPCR apparatus (Mx3000P system) using SYBR Premix ExTaqTM II (Takara Bio Inc, Otsu, Japan). Biological replicates for each set of experiments were carried out three times, and the mean value was normalized using *Actin2* as the internal control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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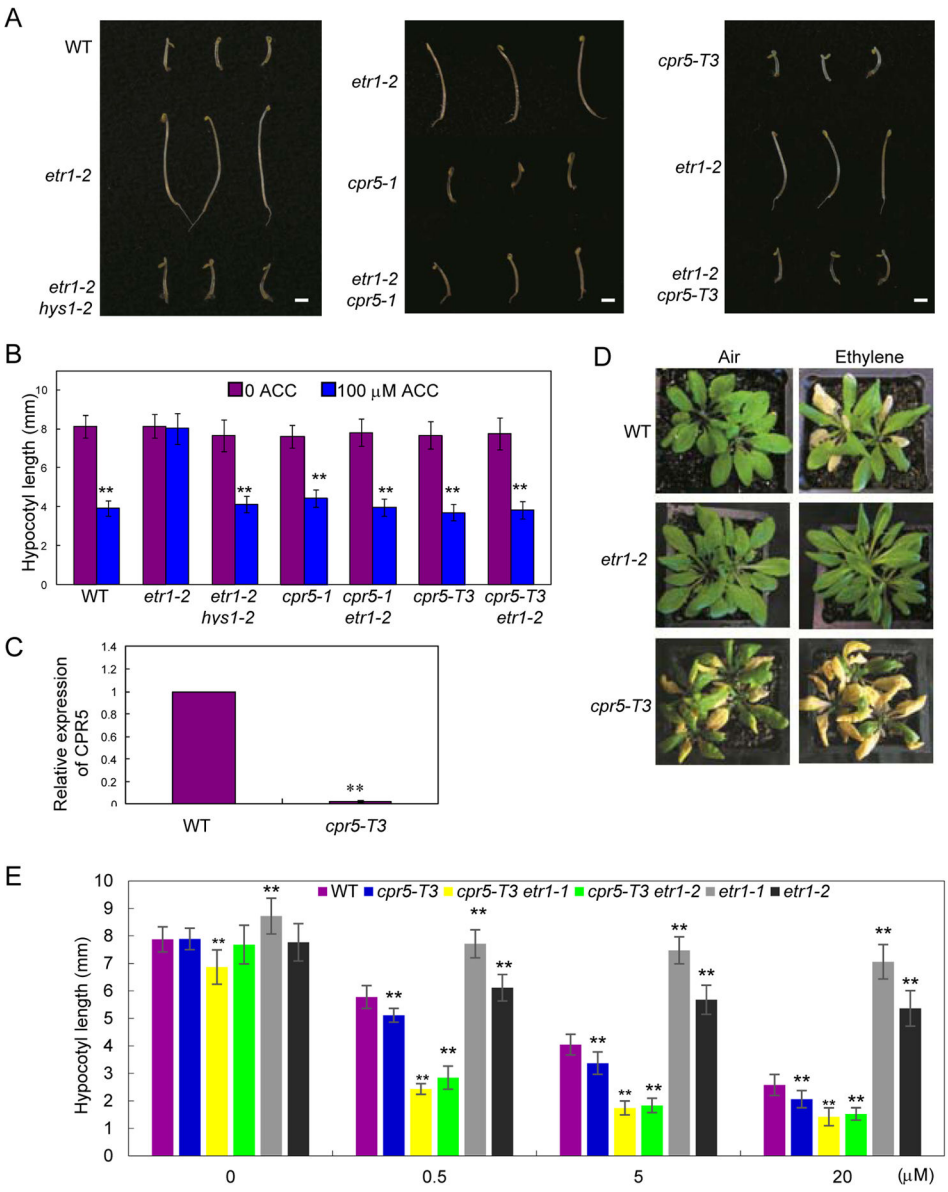

Figure 1. Ethylene sensitivity analysis of the *cpr5* mutants

(**A**) Representative 4-day-old etiolated of the wild type (WT), *etr1-2* and *etr1-2 hys1-2* germinated in the presence of the ethylene precursor ACC (100 μM). Bars= 3 mm. (**B**) Quantitative analysis of hypocotyl lengths of 4-day-old dark-grown seedlings of wild type (WT), *etr1-2*, *etr1-2 hys1-2*, *cpr5-1*, *etr1-2 cpr5-1*, *cpr5-T3*, and *cpr5-T3 etr1-2* germinated in the absence or presence of the ethylene precursor ACC (100 μM). Values are mean ±*SD*, n = 25, ** $p < 0.01$. (**C**) Relative expression of *CPR5* in the wild type (WT) and *cpr5-T3* by qRT-PCR. Values are means ±*SD*; **, $P < 0.01$. (**D**) Ethylene-induced leaf senescence in 4-week-old plants treated with or without 100 ppm of ethylene for 4 days. Leaf senescence is indicated by yellowing. (**E**) Quantitative analysis of hypocotyl lengths for the wild type (WT), *cpr5-T3*, *cpr5-T3 etr1-1*, *cpr5-T3 etr1-2*, *etr1-1*, *and etr1-2* on 1/2×MS medium containing ACC (0, 0.5, 5, 20 μM). Values are mean ±*SD*, n = 30, ** $P<0.01$.

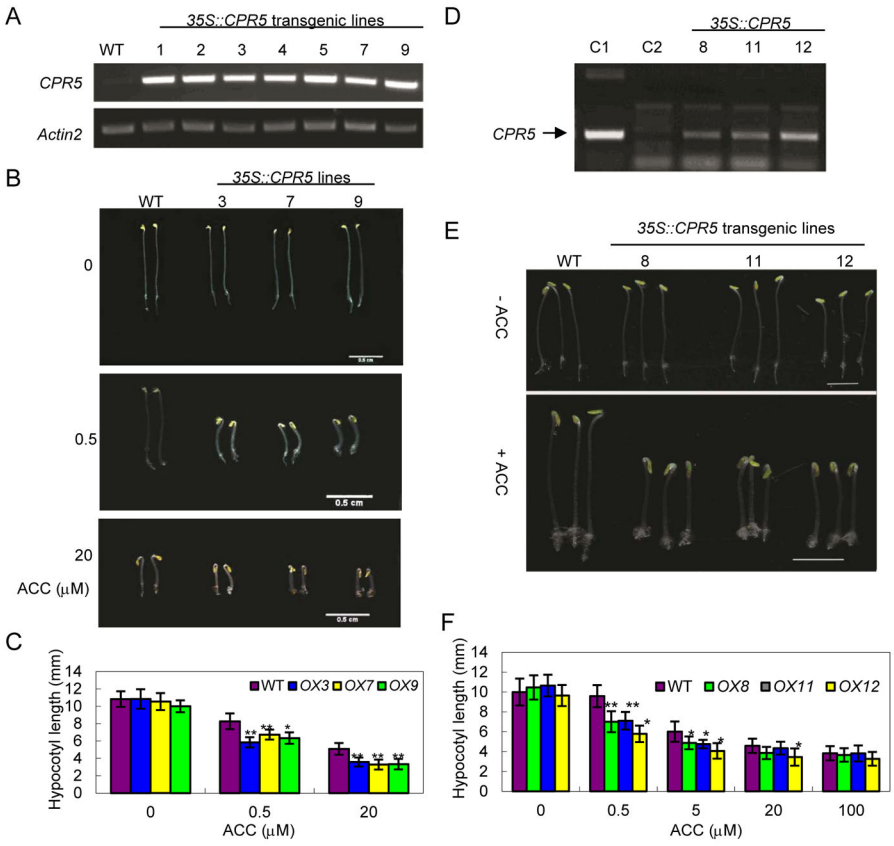

Figure 2. Analysis of transgenic plants overexpressing *CPR5*

(**A**) Semi-quantitative RT-PCR analysis for *CPR5* transcripts in wild-type (WT) and *CPR5*-overexpression lines. *Actin 2* was used as an internal control. (**B**) Ethylene sensitivity analysis showing representative 4-day old etiolated seedlings of the wild type (WT) and *CPR5*-overexpression lines treated with or without ACC (0, 0.5, 20 μM). Bars = 5 mm. (**C**) Quantitative analysis of hypocotyl lengths for the wild type (WT) and *CPR5*-overexpression lines on 1/2×MS medium containing ACC (0, 0.5, 20 μM). Values are mean ±SD, n = 20, **p<0.01. (**D**) Detection of *Arabidopsis CPR5* transcripts in the plasmid DNA control (C1), wild-type tobacco leaves (C2), and the transgenic tobacco lines (#8, 11, 12) by semi-quantitative RT-PCR. The arrow indicates the position of PCR-amplified *CPR5* fragments. (**E**) Ethylene sensitivity analysis showing representative 6-day old dark-grown seedlings the wild type (WT) and *CPR5*-overexpression transgenic tobacco lines treated with or without ACC (0.5 μM). Bars = 2 mm. (**F**) Quantitative analysis of hypocotyl lengths for the wild type (WT) and *CPR5*-overexpression transgenic tobacco lines (*OX8*, *OX11*, *OX12*) on 1/2MS medium containing ACC (0, 0.5, 5, 20, 100 μM). Values are mean ±*SD* (n = 30); **P*<005; ***P*<0.01.

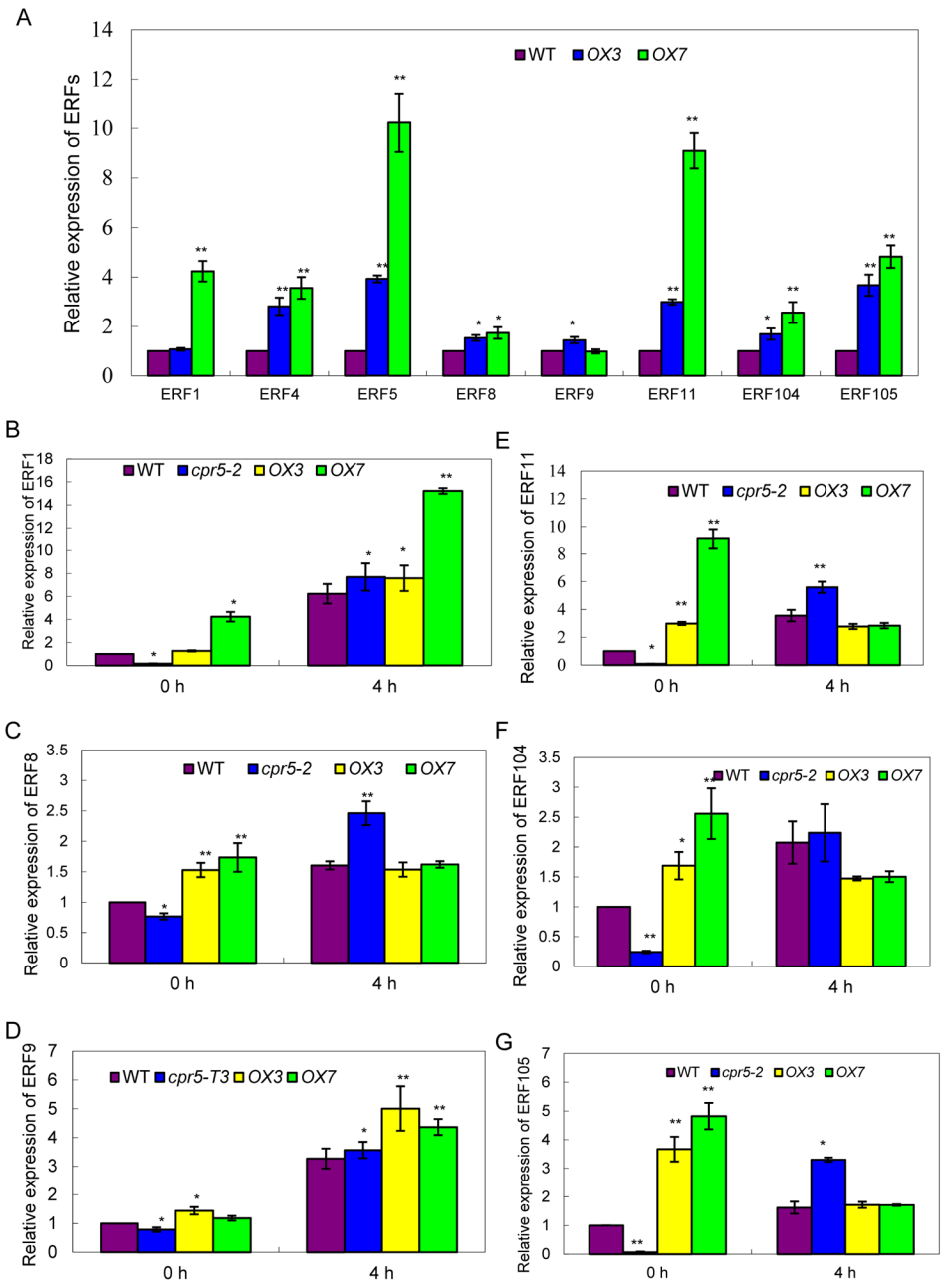

Figure 3. Relative expression of *Arabidopsis ERFs* in *cpr5-T3* and *CPR5*-overexpression lines using qRT-PCR

(**A**) Relative expression of *Arabidopsis ERF1, 4, 5, 8, 9, 11,104* and *105* in 10-day-old light-grown seedlings of the wild type (WT) and *CPR5*-overexpression lines (*OX3*, *OX7*), respectively. (**B**–**G**) Relative expression of *Arabidopsis ERF1, 8, 9, 11, 104* and *105* in 10-day-old light-grown seedlings of the wild type (WT), *cpr5-T3*, and *CPR5*-overexpression lines (*OX3*, *OX7*), respectively. Plants were grown on 1/2×MS medium for 10 days, then treated with or without ACC (100 mM) for 4 h. Values are mean ±*SD*; **P*<0.05, ** *P* <0.01.

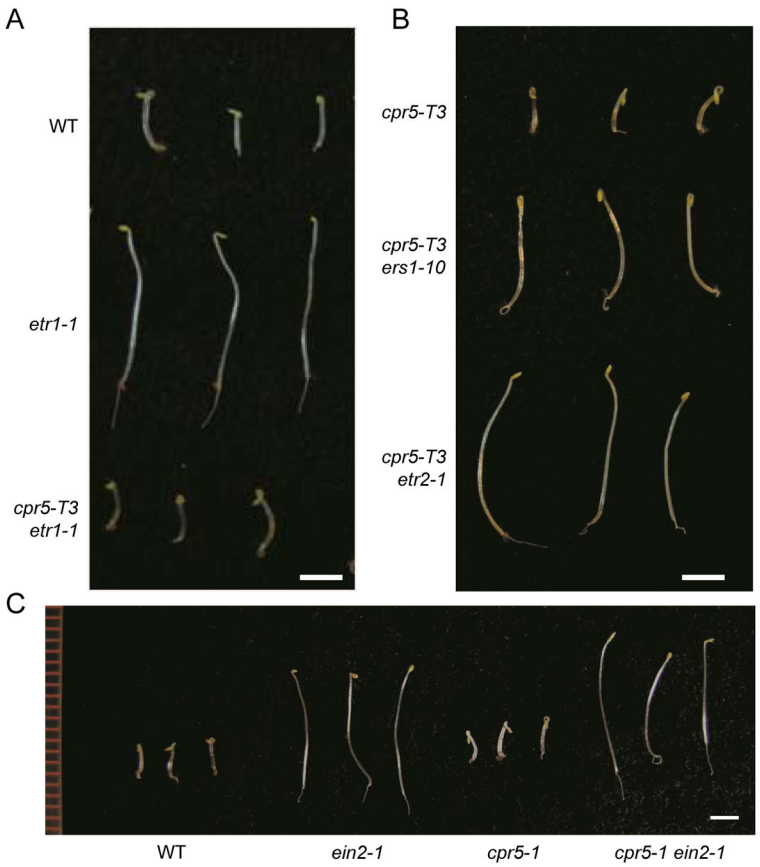

Figure 4. Ethylene "triple response" assays in *Arabidopsis* double mutants

(**A**) Comparison of 4-day-old etiolated seedlings germinated in the presence of the ethylene precursor ACC (100 μM). Three representative seedlings of the wild type (WT) and the *etr1-1* and *cpr5-T3 etr1-1* mutants are shown. (**B**) Comparison of 4-day-old etiolated seedlings of the *cpr5-T3*, *cpr5-T3 ers1-10* and *cpr5-T3 etr2-1* mutants germinated in the presence of the ethylene precursor ACC (100 μM). Three representative seedlings of the mutants are shown. (**C**) Comparison of 4-day-old etiolated seedlings of the wild type (WT) and *ein2-1*, *cpr5-1*, and *cpr5-1 ein2-1* germinated in the presence of the ethylene precursor ACC (100 μM). Scale bars = 2 mm.

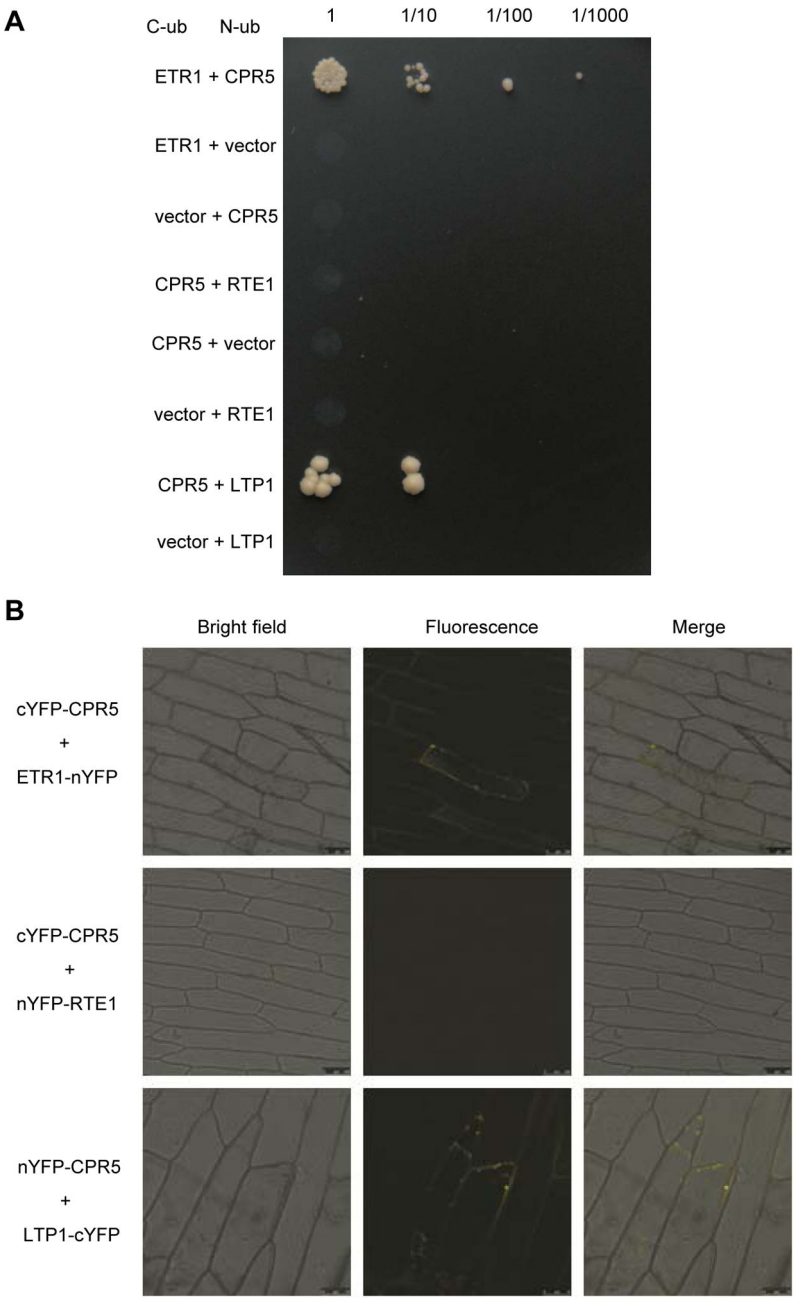

Figure 5. Molecular association of *Arabidopsis* CPR5 and ethylene receptor ETR1

(**A**) Molecular interaction of CPR5 and ETR1 in the yeast split-ubiquitin assay. CPR5 was paired with ETR1, RTE1, LTP1 or an empty vector. Yeast viability is shown on medium lacking histidine, alanine, leucine and tryptophan, while interaction is indicated by growth on the same selection medium. Undiluted and 1:10, 1:100, 1:1000 diluted liquid cultures were spotted on the indicated plates and incubated for 5 days at 30°C. (**B**) Molecular interaction of CPR5 and ETR1 in onion epidermal cells shown by BiFC assay. Constructs expressing the N- and C-terminal halves of YFP fused to the C-terminus of ETR1 or RTE1, and the N-terminus of CPR5 or LTP1, respectively, were co-infiltrated into onion epidermal

cells. YFP fluorescence was detected by laser scanning confocal microscopy at 505–530 nm. Scale bars: 75 μm or 100 μm.