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RESEARCH PAPER

Molecular association of Arabidopsis RTH with its homolog RTE1 in regulating ethylene signaling

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Abstract

The plant hormone ethylene affects many biological processes during plant growth and development. Ethylene is perceived by ethylene receptors at the endoplasmic reticulum (ER) membrane. The ETR1 ethylene receptor is positively regulated by the transmembrane protein RTE1, which localizes to the ER and Golgi apparatus. The *RTE1* gene family is conserved in animals, plants, and lower eukaryotes. In Arabidopsis, *RTE1-HOMOLOG* (*RTH*) is the only homolog of the Arabidopsis *RTE1* gene family. The regulatory function of the Arabidopsis RTH in ethylene signaling and plant growth is largely unknown. The present study shows Arabidopsis *RTH* gene expression patterns, protein co-localization with the ER and Golgi apparatus, and the altered ethylene response phenotype when *RTH* is knocked out or overexpressed in Arabidopsis. Compared with *rte1* mutants, *rth* mutants exhibit less sensitivity to exogenous ethylene, while RTH overexpression confers ethylene hypersensitivity. Genetic analyses indicate that Arabidopsis RTH might not directly regulate the ethylene receptors. RTH can physically interact with RTE1, and evidence supports that RTH might act via RTE1 in regulating ethylene responses and signaling. The present study advances our understanding of the regulatory function of the Arabidopsis RTE1 gene family members in ethylene signaling.

Key words: Arabidopsis, ethylene, receptor, RTE1 homolog, signaling.

Introduction

The gaseous hormone ethylene affects a variety of biological processes throughout a plant's lifetime including seed germination, apical hook formation, organ senescence, fruit ripening, abscission, gravitropism, and responses to various stresses (Abeles *et al.*, 1992). Great advances in our knowledge about the molecular regulation of ethylene responses and ethylene signaling have been made in the past decades by employing different biological approaches. The isolation of a series of ethylene response mutants led to identification of the key signal transduction components, including the ethylene receptors and downstream factors in the model plant *Arabidopsis thaliana*. The ethylene 'triple response' genetic screen using dark-grown Arabidopsis seedlings in the presence of ethylene was crucial in identification of the molecular regulators in ethylene responses and the signaling pathway (Bleecker *et al.*, 1988; Guzmán and Ecker, 1990; Kieber *et al.*, 1993; Roman and Ecker, 1995). There are a total of five ethylene receptors, namely ETR1, ERS1, ETR2, EIN4, and ERS2, in Arabidopsis (Chang *et al.*, 1993; Hua *et al.*, 1995; Hua and Meyerowitz, 1998; Sakai *et al.*, 1998). The ethylene receptors

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negatively regulate ethylene responses (Hua and Meyerowitz, 1998). Upon ethylene binding, the receptors inhibit the function of CTR1, and thus allow EIN2 to act as a positive regulator of the ethylene pathway (Kieber *et al.*, 1993). EIN2 facilitates the accumulation of transcription factors of the EIN3 family located in the nucleus by inhibiting the translation of F-box proteins that target EIN3 for proteolysis (Li *et al.*, 2015; Merchante *et al.*, 2015). As a master regulator of ethylene signaling, EIN3 binds to promoters of ETHYLENE RESPONSE FACTOR (ERF) genes and stimulates their transcription in an ethylene-dependent manner (Chao *et al.*, 1997; Solano *et al.*, 1998; Alonso *et al.*, 1999; Guo and Ecker, 2004). The ERF transcription factors regulate the expression of many downstream genes (Guo and Ecker, 2004). In addition to ethylene-triggered transcriptional regulation, EIN3 binding was found to modulate a multitude of downstream transcriptional cascades (Chang *et al.*, 2013).

Ethylene response and signaling are tightly regulated at different levels. EIN2 is an ethylene signal component downstream of CTR1. An early study showed that expression of the EIN2 C-terminus is sufficient to activate ethylene responses constitutively (Alonso *et al.*, 1999). Recently, it was found that the cytosolic C-terminus of EIN2 can be phosphorylated by CTR1 (Ju *et al.*, 2012). Mutations that block the EIN2 phosphorylation sites resulted in constitutive nuclear localization of the EIN2 C-terminus, conferring the constitutive activation of ethylene responses in Arabidopsis. The EIN2 C-terminus contains a putative nuclear localization signal (NLS), and removal of the NLS eliminated the ability of the C-terminus to activate ethylene signaling, suggesting that the nuclear localization of the EIN2 C-terminus is crucial for its action (Qiao *et al.*, 2012; Wen *et al.*, 2012). In addition, EIN2 can bind to the 3'-untranslated region (UTR) of *EBF1/2* mRNA in the cytoplasm and impose the translational repression of *EBF1* and *EBF2* mRNA by forming the cytoplasmic processing body (P-body) through interacting with multiple P-body factors such EIN5 (Li *et al.*, 2015).

Ethylene signaling is initiated by a family of integral membrane receptors. The ethylene receptors in Arabidopsis fall into two subfamilies based on structural similarities, ETR1 and ERS1 comprise subfamily I, and EIN4, ETR2, and ERS2 comprise subfamily II. The N-terminus of the ethylene receptors comprises an ethylene-binding domain (Schaller and Bleecker, 1995; Hall *et al.*, 2000; O'Malley *et al.*, 2005), consisting of three membrane-spanning domains localized at the endoplasmic reticulum (ER) and possibly at the Golgi apparatus (Chen *et al.*, 2002; Dong *et al.*, 2008; Grefen *et al.*, 2008). The cytosolic portion of the Arabidopsis receptors exhibits histidine and/or serine/threonine protein kinase activity *in vitro* (Gamble *et al.*, 1998; Moussatche and Klee, 2004; Voet-van-Vormizeele and Groth, 2008). However, the regulatory mechanism of ethylene receptor signaling remains largely unknown.

Ethylene receptors were shown to bind ethylene (Schaller and Bleecker, 1995; O'Malley *et al.*, 2005) with the help of a copper cofactor Cu(I) (Rodriguez *et al.*, 1999), which requires RAN1 (Hirayama *et al.*, 1999; Woeste and Kieber, 2000). When ethylene is bound, a conformational change presumably occurs within the receptor to turn off its signaling. Dominant gain-of-function mutations in any of the receptor genes lead to amino acid substitutions in the N-terminal domain that cause the receptor to signal constitutively, resulting in dominant ethylene insensitivity (Wang *et al.*, 2006).

The ethylene receptors are disulfide-linked homodimers (Schaller *et al.*, 1995; Hall *et al.*, 2000; Chen *et al.*, 2010) and form higher order multimeric complexes through non-covalent interactions (Gao *et al.*, 2008; Grefen *et al.*, 2008; Chen *et al.*, 2010). The five Arabidopsis ethylene receptors can form both homomeric and heteromeric complexes, and protein–protein interactions have been detected for all possible receptor combinations (Gao *et al.*, 2008; Grefen *et al.*, 2008; Chen *et al.*, 2010). It is thought that multiprotein complexes are the functional units for signal transduction by the ethylene receptors.

As an activator of the ethylene receptor ETR1, *RTE1* was identified in a genetic screen for suppressors of the dominant *etr1-2* receptor mutant in Arabidopsis (Resnick *et al.*, 2006). Genetic analyses suggest that Arabidopsis *RTE1* is required for *ETR1* function, but is not required for the function of the other ethylene receptors in Arabidopsis (Resnick *et al.*, 2006). RTE1 co-localizes with the ETR1 receptor at the ER and Golgi (Dong *et al.*, 2008), and RTE1 physically interacts with the ETR1 receptor (Dong *et al.*, 2010), indicating that RTE1 functions directly in controlling ethylene receptor ETR1 signaling. Arabidopsis *RTE1* encodes an integral membrane protein of 250 amino acids with three homologs in tomato, one of which was shown to regulate ethylene responses in the crop plant (Barry and Giovannoni, 2006; Klee, 2006; Ma *et al.*, 2012). In *Rosa hybrida*, it was reported that the expression of *Rh-RTH1* was responsive to ethylene, and the expression of *Rh-RTH1* also partially correlated with that of *Rh-ETR1* and *Rh-ETR3* (Yu *et al.*, 2010). Similarly, 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 modulation of ethylene responses in seedling growth and flower senescence. However, the regulatory function of the RTE1 homolog in ethylene signaling and plant growth has not been fully understood.

In Arabidopsis, *RTH* (*RTE1-Homolog*) is the only homolog of the *RTE1* gene family, encoding a protein of 231 amino acids with 44.4% identity and 61.5% similarity to RTE1 (Resnick *et al.*, 2006; Zhang *et al.*, 2012). The *RTE1* gene family is conserved in animals, plants, and lower eukaryotes, but the only known function has come from the studies of *RTE1/GR* in plant ethylene response and signaling (Barry and Giovannoni, 2006; Klee, 2006; Resnick *et al.*, 2006; Ma *et al.*, 2012). In the current study, we present the data for *RTH* gene expression, protein co-localization with the ER and Golgi apparatus, and the ethylene response phenotype when *RTH* is knocked out or overexpressed in Arabidopsis. Evidence from the study indicates that RTH plays a role in the regulation of ethylene responses and seedling growth. The *rth* knockout mutants exhibit less sensitivity to exogenous ethylene, while *RTH* overexpression confers ethylene hypersensitivity. Genetic analyses suggest that *RTH* might not directly regulate the ethylene receptors but modulates ethylene signaling

via its homolog, *RTE1*. These findings significantly advance our understanding of the regulatory function of Arabidopsis RTH via its homolog RTE1 in the ethylene signaling pathway.

Materials and methods

Genetic crosses and mutant genotyping

Mutant *rth-1* was obtained from the TILLING mutagenesis project (http://tilling.fhcrc.org:9366/search.html) in a Columbia (Col-0) background. Before performing any genetic experiments using *rth-1*, three rounds of backcrosses of the mutant with Col-0 wild-type plants were performed to remove potential unlinked mutations. To test if *rth-1* is a deficient allele, the polyclonal RTH antibody against the N-terminal region of RTH [amino acid 10–HRMMIGLSDPMKID(C)–amino acid 23] was made by Alpha Diagnostics International Inc. (www.4adi.com). The protein isolation, purification, and immunoblotting were performed as previously described (Dong *et al.*, 2010). The second allele of *rth-2* [ET9854-12, Landsberg *erecta* (L*er*) background] was obtained from an enhancer trap developed by Martienssen Lab (http://genetrap.cshl.edu) at Cold Spring Harbor Laboratory, and a T-DNA inserted immediately after the first ATG of the *RTH* gene in the mutant.

Double mutants *etr1-2 rth-1*, *etr1-2 rte1-3*, *rth-1 rte1-3*, *rth-1 ers1-3*, *rth-1 ers1-10*, *rth-1 ers2-2*, *rth-1 etr2-1*, *rth-1 ein4-1*, and *rth-1 etr1-1* were obtained by genetic crosses, and the F_2 progeny from the crosses were screened by PCR to identify homozygotes using specific PCR markers as previously described (Resnick *et al.*, 2006). The *ers2-2* transgene was selected by kanamycin resistance. The other primers used for mutant genotyping are included in Supplementary Table S1 at *JXB* online.

Membrane protein isolation and western blotting

Isolation of Arabidopsis membrane protein was previously described (Dong *et al.*, 2008, 2010). In brief, 12-day-old seedlings grown in the light were homogenized on ice in an extraction buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 10 mM EDTA, and 20% v/v glycerol) with protease inhibitor cocktail (Sigma). The homogenate was strained through Miracloth (Calbiochem-Novabiochem; http://www.emdbiosciences.com) and centrifuged at 8000 *g* for 15 min. The supernatant was centrifuged at 100 000 *g* for 30 min, and the membrane pellet resuspended in a buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 10% v/v glycerol) containing protease inhibitors.

For immunoblot analysis, membrane proteins were treated with 100 mM DTT at 37 °C for 1 h and then fractionated by SDS–PAGE on an 8% w/v polyacrylamide gel. After electrophoresis, proteins were electroblotted to a supported nitrocellulose membrane (Bio-Rad, http://www.bio-rad.com/). To detect RTH, a 1:1000 dilution of the primary rabbit polyclonal anti-RTH antibody [RTH amino acid 10–HRMMIGLSDPMKID(C)–amino acid 23] was used, followed by a 1:5000 dilution of the goat anti-rabbit–horseradish peroxidase (HRP) secondary antibody (Pierce; http://www.piercenet.com).

Construction for RTH promoter activity analysis and protein subcellular localization assay

The *RTH* promoter region consisting of a 2.9 kb genomic DNA fragment upstream of the *RTH* translation start codon (ATG) was PCR-amplified and the resulting PCR product was cloned into pEntry vector pDONR221 (Invitrogen, http://www.invitrogen.com) and verified by nucleotide sequencing. Using a Gateway recombination cloning kit (Invitrogen), the *RTH* promoter region was inserted upstream of the β-glucuronidase (GUS) reporter gene in a binary vector (pBGWFS7; Karimi *et al.*, 2002).

As previously described (Dong *et al.*, 2010), the in-frame fusion *RFP–RTH* was cloned into pDONR221 under the native *RTH* promoter, and the recombinant *pRTH::RFP-RTH* was transferred into a binary vector (pB7GWIWG2(II)) through the Gateway cloning approach (Invitrogen). The resulting construct was verified by DNA sequencing. The primers used for the vector construction are included in Supplementary Table S1.

Constructs for bimolecular fluorescence complementation (BiFC) assay

The coding sequences of the yellow fluorescent protein (YFP) halves, *cYFP* or *nYFP*, were fused to the full-length coding sequences of *RTH* and *RTE1* genes at the N-terminus of each coding sequence. To construct the binary vector expressing the *nYFP–RTE1* fusion, we first used PCR to amplify and fuse simultaneously the full-length *RTE1* coding sequence downstream of the *nYFP* sequence, which encodes the N-terminal portion of YFP (amino acids 1–349), using an *RTE1* cDNA clone and the pSPYCE-35S/pUC-SPYCE vector (Walter *et al.*, 2004) as respective templates. The *nYFP–RTE1* gene fusion fragment was cloned into the Gateway entry vector pDONR221 using the Gateway recombination system (Invitrogen). The *nYFP–RTE1* gene fusion in pDONR221 was verified by DNA sequencing and then transferred into the Gateway binary vector pH2GW7 (Karimi *et al.*, 2002) for plant transformation.

To generate the construct encoding the *cYFP–RTH* fusion, the coding sequence for the full-length *RTH* was PCR-amplified from an existing *RTH* cDNA template, cloned into pDONR221, verified by DNA sequencing, and then transferred into the binary vector pSPYNE-35S-GW (Walter *et al.*, 2004) using the Gateway cloning system (Invitrogen).

Constructs for yeast split-ubiquitin assay

For yeast split-ubiquitin assay, the DNA-binding domain fusion (bait) plasmid and transcriptional activation domain fusion (prey) plasmid were constructed. To create the bait vectors, the full-length coding sequences of *RTE1* and *RTH* or the *RTH* portions (1 – 540 bp and 163–693 bp) were each PCR-amplified and ligated into the *Sfi*I site of the bait vector pBT3-N using T4 DNA ligase. To create the prey vectors, the coding sequences of *RTE1* and *RTH* were each PCR-amplified, digested with *Sfi*I, and then cloned into the *Sfi*I sites of pPR3-N. Both inserts were verified by restriction digests and nucleotide sequencing.

Yeast two-hybrid analyses were performed as previously described (Chang *et al.*, 2014). The yeast transformants were grown overnight in minimal liquid medium lacking tryptophan and leucine (to select for the bait and prey plasmids, respectively) with shaking at 30 °C, and then serial 10-fold dilutions were spotted in 6 μl drops onto agar medium.

Plant growth and transformation

Wild-type plants of *A. thaliana* (ecotype Col-0) and tobacco (*Nicotiana benthamiana*) were grown in soil in a controlled-environment chamber at 20 °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). To select transformed plants, herbicide Basta (0.1% Finale™) was used to spray onto seedlings.

Agroinfiltration of tobacco leaves mediated by *A. tumefaciens* strain C58C1 (pCH32) was carried out as previously described (Voinnet *et al.*, 2003; Dong *et al.*, 2010). For infiltration, 50 ml cultures of *Agrobacterium* in LB broth supplemented with 10 mM MES and 20 mM acetosyringone were precipitated, washed, and resuspended in a solution (10 mM MgCl, 10 mM MES, 100 mM acetosyringone). Tobacco leaves of *N. benthamiana* from 3-week-old plants were used for infiltration.

Fluorescence microscopy

Imaging of fluoresecent proteins in tobacco leaf, Arabidopsis seedling root, or onion peel cells was conducted under a laser

scanning confocal microscope (Zeiss LSM510 or Leica TCS SP5). The excitation wavelengths for green fluorescent protein (GFP; or YFP) and red fluorescent protien (RFP) were 488 nm and 543 nm, respectively, and the emission filter wavelengths were 505–530 nm for GFP, 505–550 nm for YFP, and 560–615 nm for RFP. Pieces of tobacco leaves, fresh onion peels, or segments of 8-day-old Arabidopsis seedling roots were directly mounted in water on a glass slide for visualization. For each experiment, at least 10 different samples were scanned by laser scanning microscope. Experiments were repeated more than three times. The established fluorescent protein markers used in this study include GFP–HDEL (pVKH18En6-mGFPer) for the ER and ST–GFP (pVKH18En6-STtmd-GFP) for the Golgi apparatus (Saint-Jore *et al.*, 2002; Dong *et al.*, 2010).

RNA isolation and quantitative real-time PCR (RT-PCR) analysis

Arabidopsis plants were grown on half-strength Murashige and Skoog (MS) medium for a week in the light, and treated with 1-aminocyclopropane-1-carboxylic acid (ACC) at different concentrations in the dark. Total RNA was isolated from whole seedlings with TRIzol (Sigma), and the reverse transcription of RNA was performed using PrimeScript™ RT Enzyme Mix according to the manufacturer's recommendations (Takara Bio Inc., Otsu, Japan). The semi-quantitative and quantitative RT-PCRs were conducted with the primers given in Supplementary Table S1. Quantitative RT-PCR analysis was performed on a qPCR apparatus (Agilent, Mx3000P system) using the SYBR Premix ExTaq™ II (Takara Bio Inc.). Biological replicates for each set of experiments with three independent samples were carried out three times (n=3 × 3), and the mean value was normalized using *Actin2* or *Actin7* as the internal controls. The quantitative RT-PCR was conducted as previously described (Wang *et al.*, 2016*a*).

Statistical analysis

Statistical data were evaluated either by Student's *t*-test or Fischer's test for a multiple comparison.

Results

Arabidopsis RTH is expressed in developing seedlings and young tissues

To explore the gene expression pattern of *RTH in planta*, we constructed a *GUS* reporter gene expression cassette driven by the native *RTH* promoter. An *RTH* promoter region, consisting of a 2.9 kb genomic DNA fragment upstream of the *RTH* translation start codon, was fused with the *GUS* reporter gene, and the resulting construct was transformed into wild-type (Col-0) Arabidopsis plants by *Agrobacterium*-mediated floral bud infiltration (Clough and Bent, 1998).

Transgenic Arabidopsis plants were obtained by screening for antibiotic resistance, and >10 independent transgenic lines were obtained. Analysis of *RTH* gene expression was performed by staining for GUS activity in the transgenic lines harboring the *pRTH::GUS* construct. Different transgenic lines showed the same GUS staining pattern. Figure 1 shows the representative samples after staining for GUS activity. The Arabidopsis *RTH* gene is expressed in the developing seedlings and young tissues such as the root tip, apical hook, and shoot apex, similarly to what was observed for *RTE1* (Dong *et al.*, 2008).

Co-localization of RFP–RTH fusion proteins with ER and Golgi markers

To study whether the Arabidopsis RTH proteins localize to the ER and Golgi apparatus as previously reported for RTE1 (Zhou *et al.*, 2007; Dong *et al.*, 2008), the co-localization of RTH with the Golgi apparatus and ER using Arabidopsis marker lines that harbor either a Golgi apparatus marker (ST–GFP) or an ER marker (GFP–HDEL) (Saint-Jore *et al.*, 2002; Dong *et al.*, 2008) were examined. The coding sequence fusion of *RFP–RTH* was cloned under the native promoter of *RTH*, and the resulting construct *pRTH::RFP-RTH* was transformed into Arabidopsis transgenic lines labeled with either a Golgi marker (ST–GFP) or an ER marker (GFP-HDEL). Transgenic lines from the T_2 generation were selected, and the lines showing strong fluorescence for both RFP–RTH and a Golgi/ER marker were used for further study. Observations indicate that the RFP–RTH fusion proteins co-localize with the molecular markers for both the Golgi (ST–GFP) and ER (GFP–HDEL). Figure 2A and B shows representative images for co-localization of RFP–RTH with either marker in the epidermal cells of primary roots of Arabidopsis seedlings.

Molecular association of RTH with RTE1

We performed the yeast split-ubiquitin assay to determine whether RTH can physically interact with RTE1. The cDNA fragments of *RTH* and *RTE1* ORFs were cloned into a bait vector (pPR3-N) and a prey vector (pBT3-N), respectively. In-frame fusion of the resulting constructs was verified by DNA sequencing of the fusion genes. As shown in Fig. 3A, strong protein–protein interactions were detected in the yeast cells which co-express both RTE1 and RTH. In contrast, no colony survived on the selection medium when either of these proteins was absent. We similarly tested for, but did not detect the interaction of RTH and the ETR1 receptor using the yeast split-ubiquitin system. As a positive control, the interaction between RTE1 and ETR1 was observed (Fig. 3A). Interestingly, it was observed that RTH can also form homodimers in the cells (Fig. 3A). Moreover, we examined interaction between RTE1 and different portions of RTH (residues 1–180 and 55–231), and the experiments indicate that both the N-terminus (residues 1–54) and C-terminus of RTH (residues 181–231) where there are probably two transmembrane domains (Resnick *et al.*, 2006; Dong *et al.*, 2010) are required for the protein interaction (Fig. 3A).

We next examined this protein–protein interaction *in planta* using the BiFC assay. The coding sequences of the *YFP* halves, *cYFP* and *nYFP*, were fused to the full-length coding sequences of *RTH* and *RTE1*, respectively, at the N-terminus. RTE1 has a cytosolic N-terminus (Maggio *et al.*, 2007; Dong *et al.*, 2010), and a previous study showed that RTE1 tagged with RFP at its N-terminus is capable of rescuing an *rte1* loss-of-function phenotype (Dong *et al.*, 2008, 2010). When transiently co-expressed by cYFP–RTH and nYFP–RTE1 in onion peel cells, the fluorescent signals were readily detected (Fig. 3B). The same observations were obtained when the

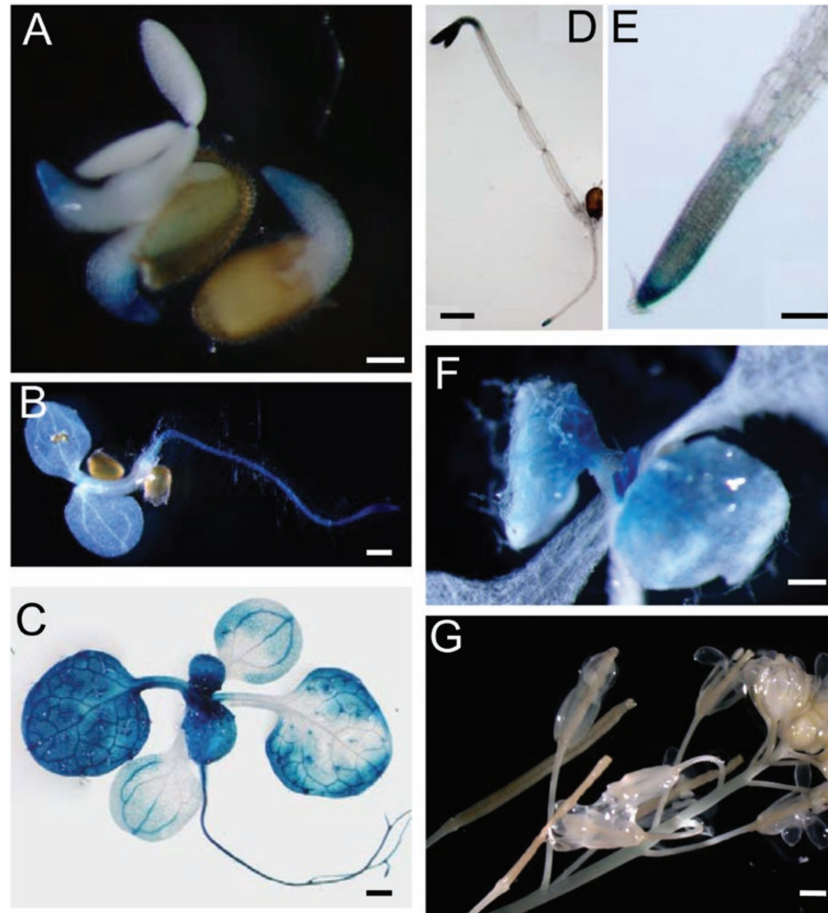

Fig. 1. *RTHpromoter–GUS* gene expression patterns. Representative images of *RTHpromoter–GUS* expression are shown in Arabidopsis plant samples: (A) 1-day-old light-grown seedlings; (B) 3-day-old light-grown seedling; (C) 9-day-old light-grown seedling; (D) 3-day-old dark-grown seedling; (E) root tip of 3-day-old dark-grown seedling; (F) shoot of 7-day-old light-grown seedling; (G) mature flowers show no GUS activity. Scale bars=1 mm (A–D, F, G), and 100 μm (E).

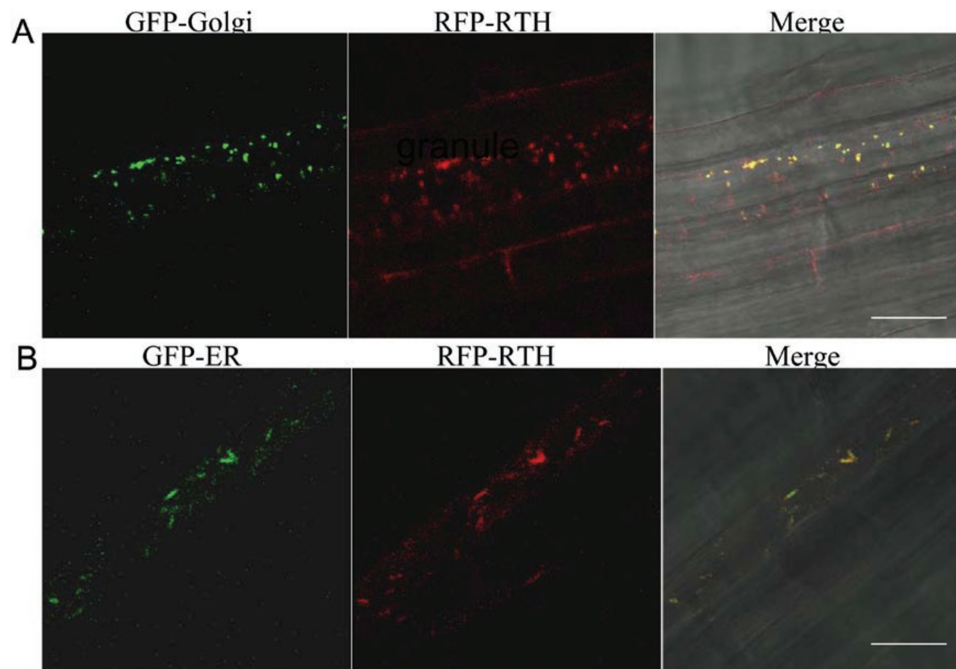

Fig. 2. Co-localization of RTH with Golgi and ER markers in plant cells. (A) Representative images showing the fluorescent Golgi marker (ST–GFP, left panel), RFP–RTH (middle panel), and merged images (right panel) in the root epidermal cells of an 8-day-old seedling co-expressing both ST–GFP and RFP–RTH. (B) Representative images showing the fluorescent ER marker (GFP–HDEL, left panel), RFP–RTH (middle panel), and merged images (right panel) in the root epidermal cells of an 8-day-old seedling co-expressing both GFP–HDEL and RFP–RTH. Scale bars=10 μm.

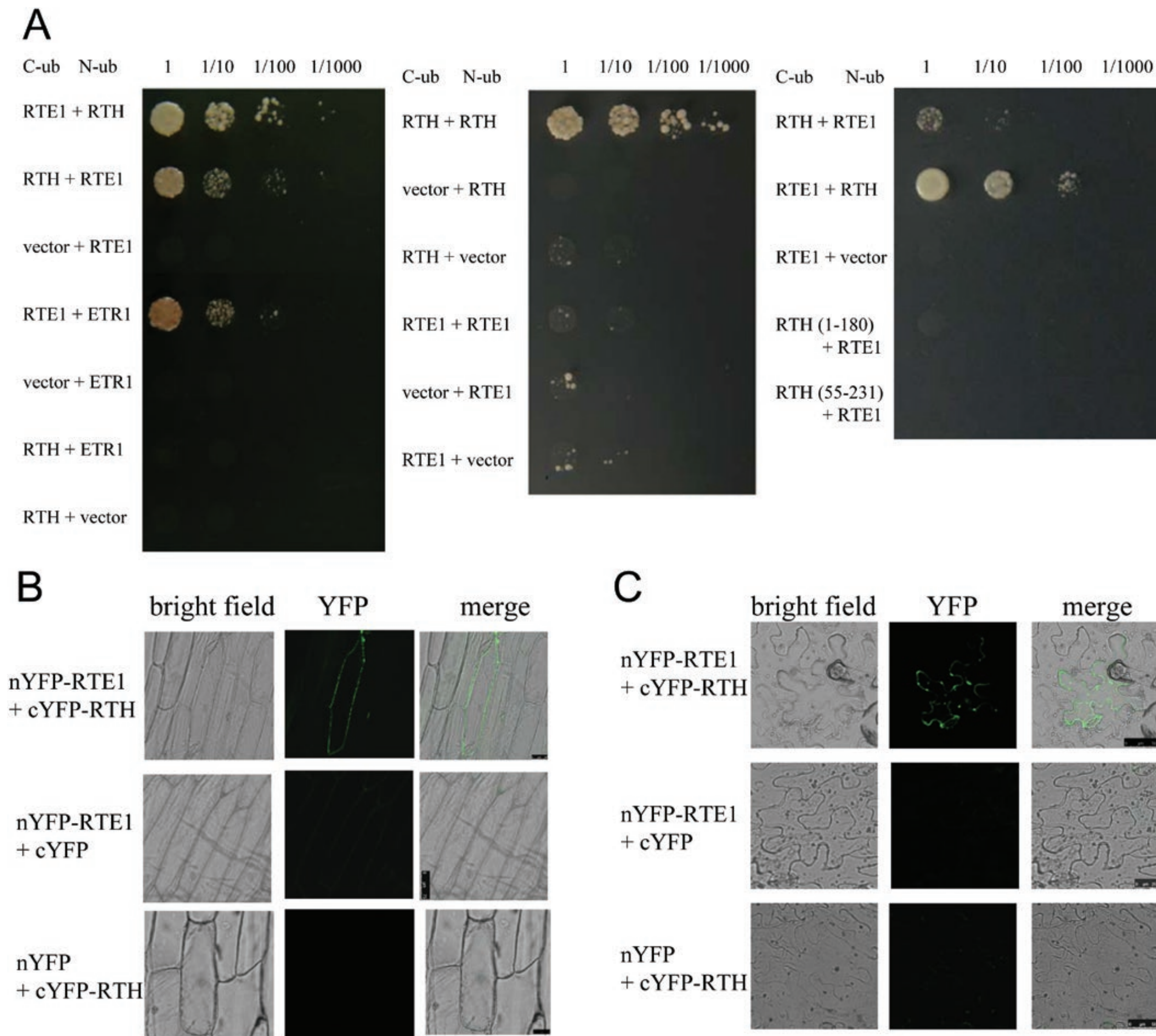

Fig. 3. Molecular association of Arabidopsis RTH with RTE1. (A) Molecular interaction between RTH and RTE1 in the yeast split-ubiquitin assay. Positive interaction is indicated by growth on medium lacking leucine, tryptophan, histidine, and alanine. Undiluted and 1:10, 1:100, and 1:1000 diluted liquid cultures were spotted on the indicated plates and incubated for 5 d at 30 °C. As a control, the ETR1 fusion paired with RTE1 or RTH was included. (B, C) Molecular interaction between RTH and RTE1 in plant cells by BiFC assay. Constructs expressing the N- and C-terminal halves of YFP fused to the N-terminus of RTH and RTE1, respectively, were co-infiltrated into onion peel cells (B) or tobacco leaf epidermal cells (C). Fluorescent YFP signals were detected by laser scanning confocal microscopy at 505–530 nm.

co-expression of RTE1 and RTH occurred in tobacco leaf epidermal cells (Fig. 3C). As expected, no signal was detected when either cYFP–RTH or nYFP–RTE1 was absent.

RTH is a positive regulator of the ethylene response in Arabidopsis seedlings

The molecular association of Arabidopsis RTH and RTE1 suggests that RTH might function similarly to its homolog RTE1 in the regulation of ethylene receptor signaling. In order to examine the effect of alteration of *RTH* levels on ethylene response, we analyzed plants in which *RTH* was genetically knocked out or overexpressed. We obtained a mutant, *rth-1*, from the TILLING project, a large-scale point mutation project in *A. thaliana* (Till *et al.*, 2003). The *rth-1* mutant carries a frameshift mutation giving an early stop codon at amino acid 108 of 231 (Q108stop). To test if *rth-1* is a deficient allele, we obtained a specific polyclonal RTH antibody from Alpha Diagnostics International Inc. (www.4adi.com). The antibody was created to target the amino terminal region of RTH [amino acid 10–HRMMIGLSDPMKID(C)–amino acid 23]. By western blotting, the wild type (Col-0) and the mutant *rte1-3* have the correct band of ~26 kDa but the same band was missing from the *rth-1* mutant (Supplementary Fig. S1). This result also indicated that the RTH antibody does not cross-react with the RTE1 protein. The second allele of the *rth* mutant (*rth-2*) in the L*er* background with a T-DNA insertion was obtained. The *RTH* transcript levels in the *rth-1*, *rth-2*, and wild-type control lines were examined by quantitative RT-PCR, and a dramatic decrease of the *RTH* transcript was observed in the mutants (Fig. 4A). In the seedling 'triple response' assay (in dark-grown seedlings), both *rth-1* and *rth-2* displayed the decreased ethylene-responsive phenotype in the presence of ACC. The mutant hypocotyls are longer than those of the wild-type controls (Fig. 4B). Interestingly, the primary roots of both *rth-1* and *rth-2* mutants are longer than those of the wild type, and more lateral roots developed from the *rth* mutants when grown in the light (Fig. 4C).

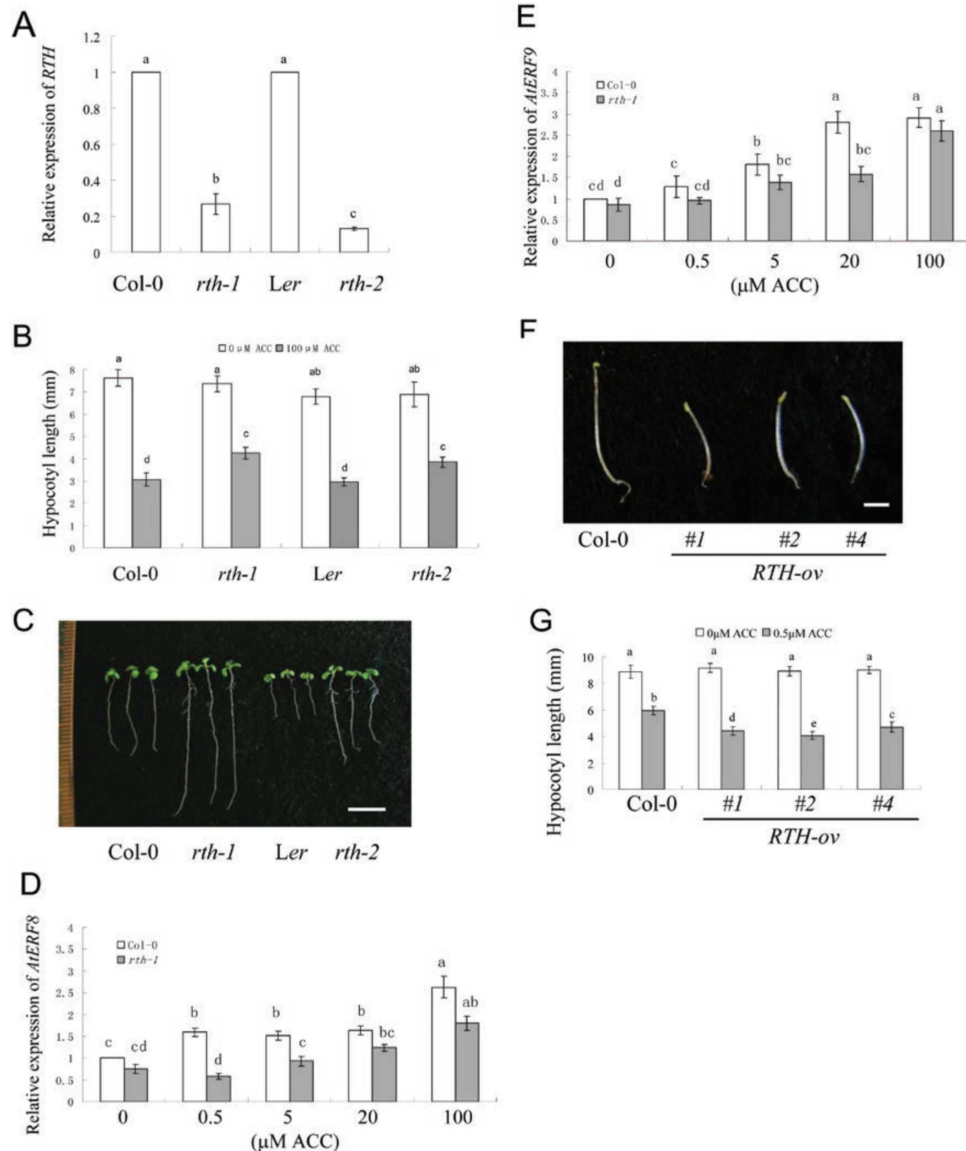

Fig. 4. Analysis of Arabidopsis *rth-1* mutants. (A) The relative expression of *RTH* in Col-0, *rth-1*, L*er*, and *rth-2* by qRT-PCR. Values are means ± SD. Significant differences between measurements (P<0.05) are indicated by different letters above the bars. (B) Hypocotyl length measurements for the 4-day-old dark-grown seedlings of Col-0, *rth-1*, L*er*, and *rth-2* germinated in the presence of the ethylene precursor ACC (100 μM). For each genotype, values are means ± SD (n=25). *P<0.05. (C) Comparison of 11-day-old light-grown seedlings of Col-0, *rth-1*, L*er*, and *rth-2*. Three representative seedlings are shown. Scale bar=5 mm. (D, E) The relative expression of *AtERF8* and *AtERF9* in Col-0 and *rth-1* by qPCR. The seedlings were germinated on the medium with different concentrations of ACC (0, 0.5, 5, 20, and 100 μM). Values are means ± SD. Significant differences between measurements (P<0.05) are indicated by different letters above the bars. (F) Comparison of 4-day-old dark-grown seedlings germinated in the presence of the ethylene precursor ACC (0.5 μM). The representative seedlings of the wild type (WT) and three *RTH*-overexpressing lines (*RTH-ov*, lines #1, #2, and #4) are shown. Scale bar=1 mm. (G) Hypocotyl length measurements for the 4-day-old dark-grown seedlings in the wild type (WT) and three *RTH*-overexpressing lines (*RTH-ov*, lines #1, #2, and #4) germinated in the presence of the ethylene precursor ACC (0, 0.5 μM). For each genotype, the mean value ± SD is shown for >30 seedlings. Significant differences between measurements (P<0.05) are indicated by different letters above the bars.

The expression levels of the downstream ERF transcription factors were analyzed in the *rth-1* mutant by using quantitative RT-PCR. In the presence of ACC, the ethylene-induced transcription of *AtERF8* and *AtERF9* was well induced in both *rth-1* and wild-type plants. The experiments show that the expression levels of the examined *ERF* genes (*AtERF8* and *AtERF9*) in the *rth-1* mutant are lower than those of the wild type when different concentrations of ACC were used (0, 0.5, 5, 20, and 100 μM) (Fig. 4D, E).

To generate *RTH* overexpression transgenic plants, the full-length cDNA of the *RTH* ORF was PCR-amplified and the resulting product was cloned under the strong *Cauliflower mosaic virus* (CaMV) 35S promoter. The resulting binary construct, *p35S::RTH*, was transferred into *Agrobacterium* for floral dip transformation of *A. thaliana* plants as described (Clough and Bent, 1998). Seven independent transgenic lines from the T_2 generation were obtained and the *RTH* transcript levels were examined by RT-PCR. Three lines showing high levels of *RTH* transcripts were used for examination of the ethylene 'triple response'. Under exposure to saturated ethylene treatment (100 μM ACC), the etiolated seedlings of the *RTH*-overexpressing lines showed no significant difference

compared with the wild type, and all of them displayed the typical 'triple response' phenotype (Supplementary Fig. S2). Interestingly, under low doses of exogenous ACC (0.5 µM), the *RTH* overexpression lines displayed an enhanced seedling 'triple response' phenotype, as shown in Fig. 4F and G. This result indicates that *RTH* overexpression conferred increased sensitivity to ethylene.

Arabidopsis RTH might function via RTE1 in regulating ethylene signaling

To test whether RTH regulates ethylene receptor ETR1 signaling, we created the double mutant *etr1-2 rth-1*. The F_2 progeny from the genetic crosses were genotyped by molecular markers for both *etr1-2* and *rth-1*, and the double mutant *etr1-2 rth-1* was identified from the F_2 progeny. An ethylene response assay of the etiolated seedlings was performed to compare it with that of the double mutant *etr1-2 rte1-3*, which was previously reported (Resnick *et al.*, 2006). Interestingly, the double mutant *etr1-2 rth-1* exhibited a different phenotype from that of the double mutant *etr1-2 rte1-3* (Fig. 5A). The *rth* mutant did not suppress the ethylene insensitivity conferred by *etr1-2* in the double mutant. This observation indicates that RTH might function differently from RTE1.

We next investigated whether *rth-1* can affect the ethylene response in other ethylene receptor mutants. Genetic crosses of *rth-1* with the mutants *etr1-1*, *etr2-1*, *ein4-1*, *ers1-3*, *ers1-10*, *ers2-2*, and *ein4-1* were performed, and each double mutant was obtained. After confirmation of the resulting double

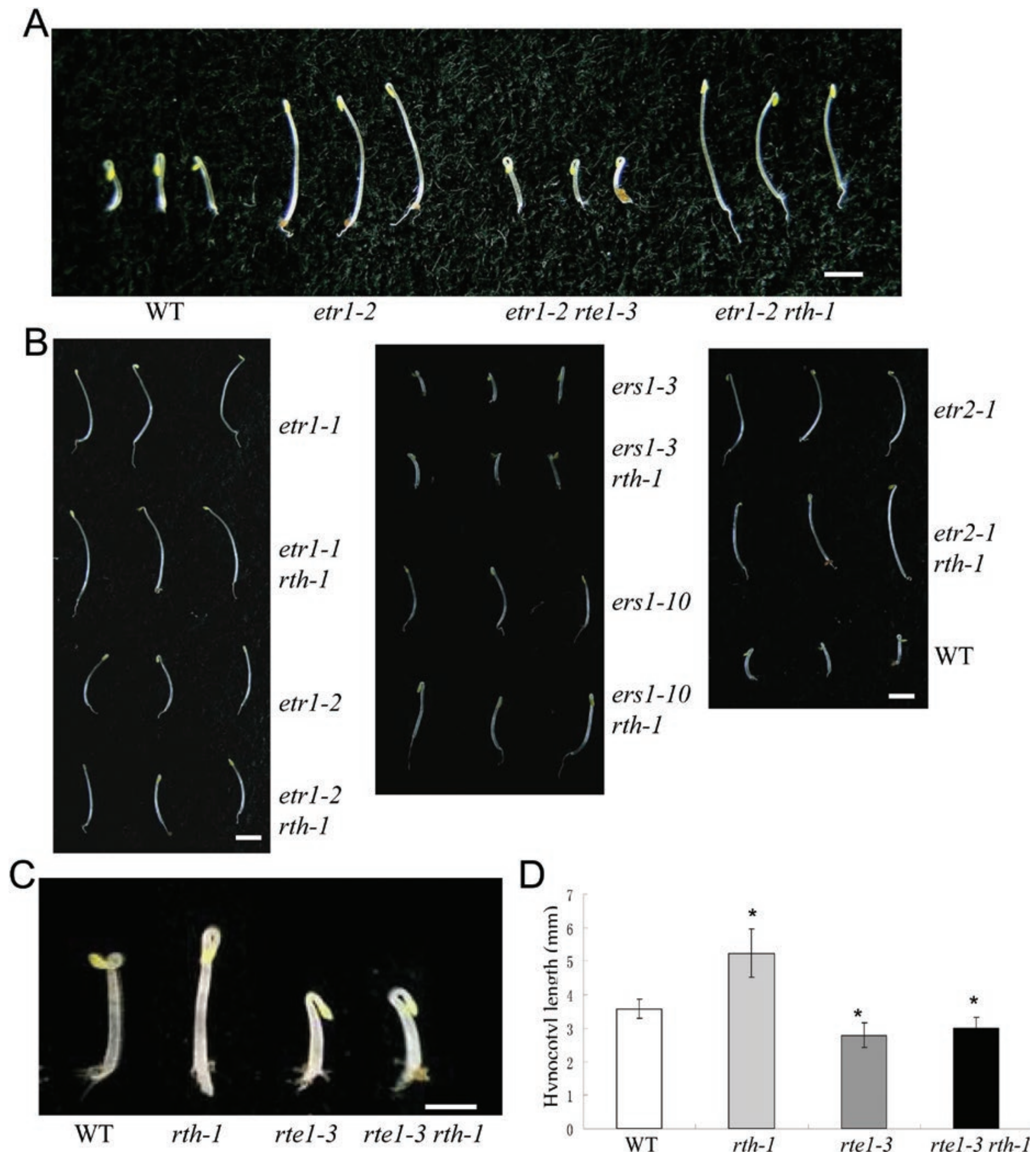

Fig. 5. Ethylene 'triple response' assays in double mutants. (A) Comparison of 4-day-old dark-grown seedlings germinated in the presence of the ethylene precursor ACC (100 µM). Three representative seedlings of the wild type (WT), *etr1*-2, *etr1-2 rte1*-3, and *etr1-2 rth-1* are shown. Scale bar=2 mm. (B) Comparison of 4-day-old dark-grown seedlings of different double mutants germinated in the presence of the ethylene precursor ACC (100 µM). Three representative seedlings of the mutants are shown. Scale bar=2 mm. (C) Comparison of 4-day-old dark-grown seedlings germinated in the presence of the ethylene precursor ACC (100 µM). Scale bar=2 mm. (D) Hypocotyl length measurements for the 4-day-old dark-grown seedlings in (C). For each genotype, the mean value ± SD is shown for >30 seedlings. **P*<0.05.

mutants by different molecular markers, suppression or exaggeration of the ethylene response of the double mutants was examined. Seedling 'triple response' analyses revealed that none of the double mutants showed significant suppression or exaggeration among the double mutants. Among them, some etiolated seedlings of the double mutants on ACC medium (100 μM) are shown in Fig. 5B. The results suggest that RTH might not directly regulate the ethylene receptors.

To examine whether RTH functions through RTE1 in regulating ethylene signaling, the double null *rth-1 rte1-3* was generated, thus removing both of the RTE family members from the plant. Interestingly, the seedling 'triple response' phenotype of the double mutant *rth-1 rte1-3* closely resembled that of the *rte1* mutant, and both the double mutant *rth-1 rte1-3* and the *rte1-3* mutant showed ethylene hypersensitivity when ACC (100 μM) was added in the medium (Fig. 5C, D).

To examine further whether the *RTH* overexpression phenotype is dependent on RTE1, transgenic plants were generated in which *RTH* is overexpressed in *rte1-3* and *RTE1* is overexpressed in *rth-1*. The transgenic lines from each transformation were obtained, and the transcripts levels of *RTH* or *RTE1* were examined in the *rte1-3* or *rth-1* mutants by RT-PCR (Fig. 6A, B, E). The lines showing high levels of *RTH* or *RTE1* transcripts were used for the ethylene response analyses. When *RTH* was overexpressed in the *rte1-3* mutant, the enhanced ethylene sensitivity by *rte1-3* was slightly promoted (Fig. 6D). In contrast, when *RTE1* is overexpressed in *rth-1*, a slight increase of ethylene insensitivity in the transgenic lines was observed (Fig. 6F, G).

Discussion

In Arabidopsis, RTE1 and RTH are the only members of this gene family, which is highly conserved in animals, plants, protists, and some fungi (Klee, 2006; Resnick *et al.*, 2006). So far the only functional insight into this protein family comes from ethylene signaling in plants, and the only known target of RTE1 action is the ETR1 ethylene receptor (Barry and Giovannoni, 2006; Resnick *et al.*, 2006, 2008; Zhou *et al.*, 2007). RTE1 is a positive regulator of the ethylene receptor ETR1. RTE1 co-localizes with ETR1 receptor at the ER and Golgi, 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 unclear whether Arabidopsis RTH acts in the same way as RTE1 in regulating ethylene receptor signaling.

As shown in Fig. 5A, *rth* did not act like *rte1* in the suppression of the ethylene insensitivity conferred by *etr1-2* in the presence of ACC, suggesting that Arabidopsis RTH might function differently from RTE1. To explore the regulatory function of RTH in ethylene response and signaling, various approaches were applied in the present study. Knockout of *RTH* in Arabidopsis (*rth-1*, *rth-2*) exhibits less sensitivity to exogenous ethylene, while *RTH* overexpression conferred ethylene hypersensitivity under a low dosage of ACC (0.5 μM) (Fig. 4B, F, G). This result was in contrast to that of *rte1-3*, which showed a slight hypersensitive phenotype in responding to exogenous ethylene (Fig. 5C; see also Resnick *et al.*, 2006). In support of the lower ethylene sensitivity conferred by *rth-1*, the quantitative measurements of expression of the downstream *ERF* genes (*AtERF8* and *AtERF9*) by RT-PCR showed that the examined *ERF* gene transcript levels in the *rth-1* mutant are lower than those of the wild type (Fig. 4D, E). Further analysis using the double mutants made from *rth-1* with the other ethylene signaling mutants (*etr1-1*, *etr2-1*, *ein4-1*, *ers1-1*, *ers1-3*, *ers1-10*, *ers2-2*, and *ein4-1*) showed that none of the ethylene insensitivity or ethylene exaggeration responses among the mutants was significantly affected by *rth-1* (Fig. 5B), suggesting that Arabidopsis RTH might not directly regulate the ethylene receptors. However, it could not be excluded that RTH may still function through the ethylene receptors.

To study the molecular mechanism by which RTH regulates ethylene response and signaling, protein–protein interaction assays were performed. As shown in Fig. 3, the yeast split-ubiquitin (Fig. 3A) and *in planta* BiFC (Fig. 3B, C) assays were performed for the molecular association of RTH with RTE1. The observation that RTH physically interacts with RTE1 suggests that RTH might function with RTE1 in the same pathway. The seedling 'triple response' phenotype of the double mutant *rte1-3 rth-1* closely resembles that of *rte1*, as observed (Fig. 5C, D). In addition, the transgenic plants in which *RTH* is overexpressed in *rte1-3* display enhanced ethylene sensitivity, suggesting that RTH acts upstream of RTE1 in regulating ethylene signaling (Fig. 6C, D). As expected, *RTH* and *RTE1* share very similar gene expression patterns by which they are highly expressed in the developing seedling and young tissues (Fig. 1; Dong *et al.*, 2008), and both of them localize to the Golgi and ER organelles (Fig. 2; Dong *et al.*, 2008).

Recently, it was shown that the Arabidosis RTE1 also interacts with cytochrome b_5 (Chang *et al.*, 2014), and a lipid transfer protein LTP1 (Wang *et al.*, 2016*b*). The Arabidopsis *atcb5* mutants show increased ethylene sensitivity, while overexpression of *AtCb5-D* confers decreased ethylene sensitivity. Being similar to *atcb5*, the *ltp1* knockout exhibits increased sensitivity to exogenous ACC, while LTP1 overexpression confers decreased sensitivity to ACC. It appears that both AtCb5 and LTP1 play positive roles in ethylene signaling and responses, probably by participating in the protein complex which is involved in the regulation of ethylene signal transduction of the ETR1 receptor via RTE1. However, evidence from this study suggests that RTH plays a different role in the regulation of ethylene signaling and responses, compared with those of AtCb5 and LTP1. Both *rth-1* and *rth-2* mutants exhibit less sensitivity to the exogenous ACC, while RTH overexpression confers ethylene hypersensitivity (Fig. 4B, F, G). Currently, it is not known how exactly RTH regulates ethylene signaling via RTE1.

It is worth noting that knockout of *RTH* promotes seedling primary growth and lateral root initiation (Fig. 4C), suggesting that *RTH* is likely to be involved in other cellular activities in addition to ethylene signaling regulation. Unfortunately, we failed to

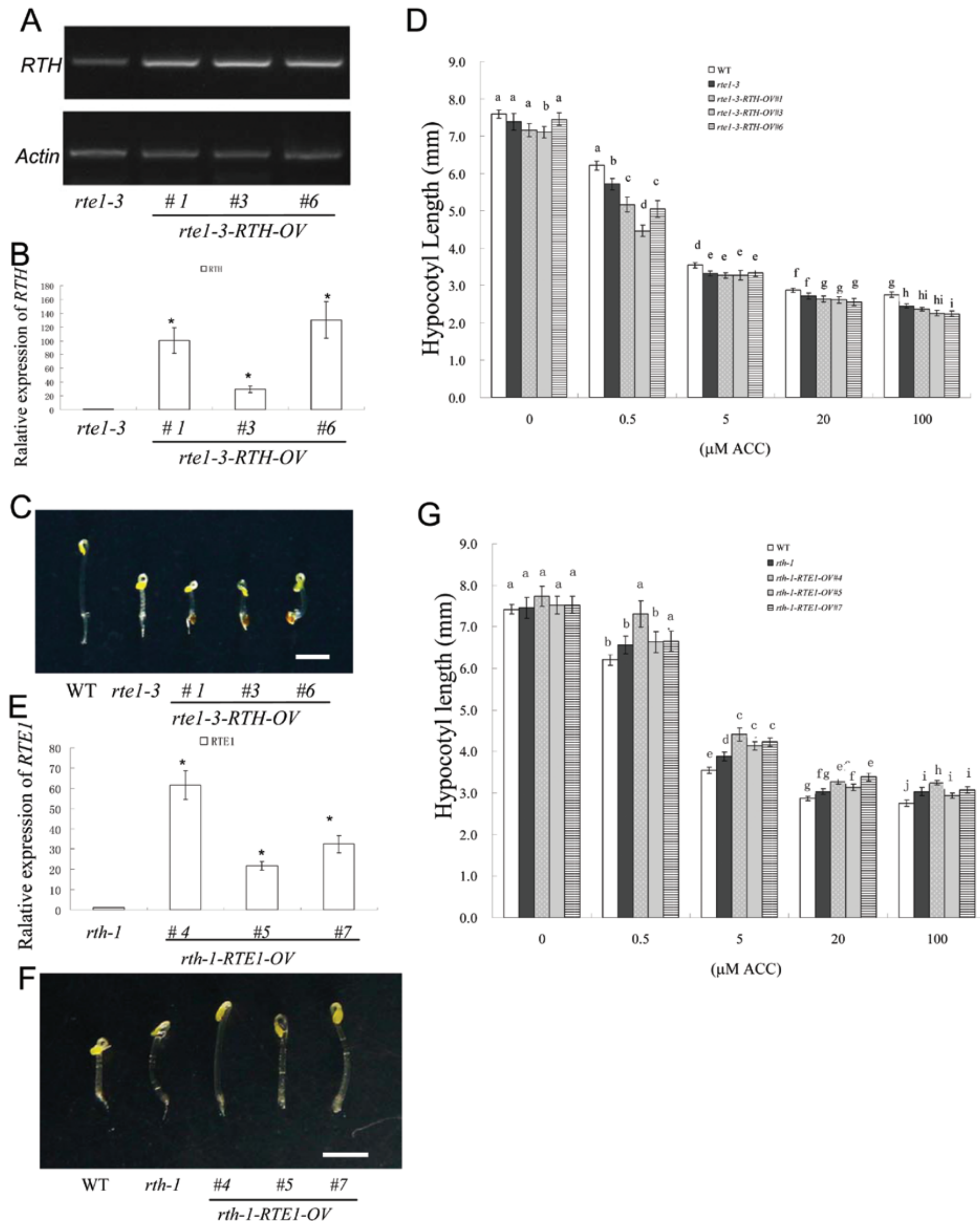

Fig. 6. RTH probably regulates ethylene signaling via RTE1. (A) Semi-quantitative RT-PCR analysis for *RTH* transcripts in *rte1-3* and *rte1-3-RTH-OV* lines. *Actin2* was used as an internal control. (B) The relative expression of *RTH* in *rte1-3* and *rte1-3-RTH-OV* lines by qRT-PCR. Values are means ± SD; **P*<0.05. (C) Representative seedlings of the wild type (WT), *rte1-3*, and *rte1-3-RTH1-OV* transgenic lines germinated in the presence of the ethylene precursor ACC (100 μM) in darkness. Scale bar=2 mm. (D) The *RTH* overexpression in *rte1-3* does not change the ethylene sensitivity in *rte1-3*. The 4-day-old etiolated seedlings of the wild type, *rte1-3*, and three transgenic lines of *RTH-OV* (#1, #3, and #6) in the *rte1-3* background were germinated on medium with different concentrations of ACC (0, 0.5, 5, 20, and 100 μM). Quantitative analysis of hypocotyl lengths is shown. Significant differences between measurements (*P*<0.05) are indicated by different letters above the bars (*n*=25). (E) The relative expression of *RTE1* in *rth-1* and *rth-1-RTE1-OV* lines by qPCR. Values are means ± SD; **P*<0.05. (F) Representative 4-day-old etiolated seedlings of the wild type (WT), *rth-1*, and *rth-1-RTE1-OV* transgenic lines germinated in the presence of the ethylene precursor ACC (100 μM). Scale bar=2 mm. (G) Ethylene sensitivity in *RTE1* overexpression lines. The 4-day-old etiolated seedlings of the WT, *rth-1*, and three transgenic lines of *RTE1-OV* (#4, #5, and #7) in the *rth-1* background were germinated on the medium with different concentrations of ACC (0, 0.5, 5, 20, and 100 μM). Quantitative analysis of hypocotyl lengths is shown. Significant differences between measurements (*P*<0.05) are indicated by different letters above the bars (*n*=25).

detect any significant effect of the *rth* mutations on the PIN protein location patterns in the cells of seedling primary roots (data not shown). The regulatory function of RTH and RTE1 proteins in root growth and development needs further study.

In summary, the present study provides evidence showing that Arabidopsis *RTH* and its homolog *RTE1* share a similar gene expression pattern, localize to the Golgi and ER, and physically interact in plant cells. Although RTH functions differently from RTE1 in the regulation of ethylene receptor ETR1 signaling, evidence supports that RTH acts via RTE1 in regulating ethylene responses and signaling. Taken together, the present study advances our understanding of the regulatory function of the *RTE1* gene family members in the ethylene signaling pathway.

Supplementary data

Supplementary data are available at *JXB* online.

Fig. S1. RTH protein analysis in *rth-1* and *rte1-3* mutants.

Fig. S2. Analysis of Arabidopsis *RTH-ov* seedlings.

Table S1. Primers for mutant genotyping, gene expression analysis, and vector construction.

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