Molecular Association of the Arabidopsis ETR1 Ethylene Receptor and a Regulator of Ethylene Signaling, RTE1*§

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The plant hormone ethylene plays important roles in growth and development. Ethylene is perceived by a family of membrane-bound receptors that actively repress ethylene responses. When the receptors bind ethylene, their signaling is shut off, activating responses. REVERSION-TO-ETHYLENE SENSITIVITY (RTE1) encodes a novel membrane protein conserved in plants and metazoans. Genetic analyses in Arabidopsis thaliana suggest that RTE1 promotes the signaling state of the ethylene receptor ETR1 through the ETR1 N-terminal domain. RTE1 and ETR1 have been shown to co-localize to the endoplasmic reticulum (ER) and Golgi apparatus in Arabidopsis. Here, we demonstrate a physical association of RTE1 and ETR1 using in vivo and in vitro methods. Interaction of RTE1 and ETR1 was revealed in vivo by bimolecular fluorescence complementation (BiFC) in a tobacco cell transient assay and in stably transformed Arabidopsis. The association was also observed using a truncated version of ETR1 comprising the N terminus (amino acids 1–349). Interaction of RTE1 and ETR1 was confirmed by co-immunoprecipitation from Arabidopsis. The interaction occurs with high affinity (Kd, 117 nM) based on tryptophan fluorescence spectroscopy using purified recombinant RTE1 and a tryptophan-less version of purified recombinant ETR1. An amino acid substitution (C161Y) in RTE1 that is known to confer an ETR1 loss-of-function phenotype correspondingly gives a nearly 12-fold increase in the dissociation constant (Kd, 1.38 μM). These findings indicate that a high affinity association of RTE1 and ETR1 is important in the regulation of ETR1.

The gaseous plant hormone ethylene is important in regulating many aspects of growth and development, including fruit ripening, senescence, abscission, and stress responses (1). There are a number of known components in ethylene signalization that form a pathway starting with the perception of ethylene and leading to gene expression changes (2, 3). Arabidopsis thaliana has five homologous ethylene receptors with sequence similarity to histidine protein kinase receptors (4–7). The receptors appear to be largely redundant in ethylene signaling, although the ETR1 ethylene receptor has a more predominant role (8–10). The N terminus of the ethylene receptors comprises an ethylene-binding domain (11–13) consisting of three membrane-spanning domains localized at the endoplasmic reticulum (ER) (14–16) and the Golgi apparatus (16). The cytosolic portion of the receptors exhibits histidine and/or serine/threonine protein kinase activity in vitro (17, 18) and control of autokinase activity by ethylene was demonstrated by in vitro phosphorylation studies using purified full-length ETR1 (19). However, the molecular mechanism of ethylene receptor signaling is still unknown, particularly as protein kinase activity appears to be largely dispensable for ethylene receptor signaling (8, 9).

The ethylene receptors are negative regulators of ethylene response, repressing responses in the absence of ethylene (10, 20) with the N-terminal domain controlling the signaling state of the receptor (21). 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 encode amino acid substitutions in the N-terminal domain that cause the receptor to signal constitutively, resulting in dominant ethylene insensitivity (21).

The ethylene receptors are disulfide-linked homodimers (12, 22, 23) and form higher order multimeric complexes through non-covalent interactions (15, 23, 24). The five Arabidopsis ethylene receptors can form both homomeric and heteromeric complexes (23), and protein-protein interactions have been detected for all possible receptor combinations (15, 24). It is thought that higher order clustering allows for conformational changes within one receptor to be propagated to other receptors in the cluster, providing a mechanism for signal amplification.

The Arabidopsis REVERSION-TO-ETHYLENE SENSITIVITY 1 (RTE1) gene is a positive regulator of ETR1 that was

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¶ The abbreviations used are: RTE, REVERSION-TO-ETHYLENE SENSITIVITY; BiFC, bimolecular fluorescence complementation; CaMV, Cauliflower Mosaic Virus; LB, Luria-Bertani; MS, Murashige & Skoog salt formula; YFP, yellow fluorescent protein.
identified in a genetic screen for suppressors of the dominant
\textit{etr1-2} receptor mutant (25). \textit{etr1} loss-of-function mutants display ethylene hypersensitivity similar to \textit{etr1} loss of function mutants. Interestingly, some dominant \textit{etr1} alleles, such as \textit{etr1-2}, are highly dependent on \textit{RTE1} to confer ethylene insensitivity, while other dominant \textit{etr1} alleles, such as \textit{etr1-1}, are largely or entirely \textit{RTE1} independent (26). All of these dominant alleles encode amino acid substitutions within the ETR1 N-terminal domain, leading to speculation that the basis for \textit{RTE1} dependence/independence may be related to the specific conformation of the ETR1 N terminus. Unexpectedly, \textit{RTE1} is highly specific for \textit{ETR1} and has no apparent role in the signaling of the four other \textit{Arabidopsis} ethylene receptors (25, 27, 28).

\textit{RTE1} encodes a novel protein carrying two to four predicted transmembrane domains (25). \textit{RTE1} is highly conserved in plants and metazoans. Homologs all carry a domain of unknown function called DUF778, which is also found in some protists and fungi. The only functional insight into this protein family comes from ethylene signaling in plants, and the only known target of \textit{RTE1} action is the ETR1 ethylene receptor. The \textit{Arabidopsis} genome carries a second copy of \textit{RTE1}, called \textit{RTE1-HOMOLOG (RTH)}, but \textit{RTH} does not appear to play the same role as \textit{RTE1} in ethylene signaling.\textsuperscript{3} Overexpression of a tomato \textit{RTE1} homolog, \textit{GREEN-RIPE}, confers inhibition of tomato fruit ripening along with other ethylene-insensitive phenotypes (29). Likewise, \textit{RTE1} overexpression in \textit{Arabidopsis} confers ethylene insensitivity (25, 28). This insensitivity is partially blocked by an \textit{etr1}-null mutation, but is restored by expression of the ETR1 N terminus (residues 1–349), indicating that the ETR1 N terminus (residues 1–349) is the downstream target of \textit{RTE1} action (28).

\textit{RTE1} is found in the microsomal fraction\textsuperscript{3} and co-localizes with \textit{ETR1} at the ER and Golgi apparatus (16). This report demonstrates a physical interaction of the \textit{RTE1} protein with the \textit{ETR1} receptor both \textit{in vivo} and \textit{in vitro}, examining the specificity of the interaction and the effects of \textit{etr1} and \textit{etr1} mutations. This work advances our understanding of \textit{RTE1} function and indicates that a physical association of \textit{RTE1} and the \textit{ETR1} receptor may be important for the regulation of \textit{ETR1} in ethylene signaling.

**EXPERIMENTAL PROCEDURES**

\textit{Plant Growth and Plant Transformation—Wild-type \textit{Arabidopsis thaliana} (ecotype Columbia (Col-0)) and \textit{Nicotiana benthamiana} were grown in soil under 16-h light/8-h dark in a controlled environment chamber at 20 °C under white fluorescent light. For protein extraction, seedlings were germinated on 1 × MS plates for 3 days in the dark at 20 °C. Stably transformed \textit{Arabidopsis} plants were generated by the floral dip infiltration method mediated by \textit{Agrobacterium tumefaciens} strain GV3101 (30). To select for transformants, seedlings were either grown on 1 × MS plates containing hygromycin 25 mg/liter or grown in soil and sprayed with Basta (0.033% Liberty Herbicide, Bayer Cropscience).}

\textsuperscript{3} C. Chang, unpublished data.

Agroinfiltration of tobacco leaves was carried out as previously described (31). \textit{A. tumefaciens} strain C58C1 (pCH32) was grown in LB-broth supplemented with 5 mg/liter tetracycline and 100 mg/liter rifampicin. To enhance transgene expression, we co-infiltrated with \textit{Agrobacterium} carrying the p19 suppressor of gene silencing (31) grown in LB-broth supplemented with 50 mg/liter kanamycin. For infiltration, 50-ml cultures of \textit{Agrobacterium} in LB-broth supplemented with 10 mM MES and 20 mM acetylsyringone were precipitated, washed, and resuspended in a solution containing 10 mM MgCl\textsubscript{2}, 10 mM MES and 100 mM acetylsyringone. Tobacco leaves from 3-week-old plants were used for infiltration. Ten plants (two leaves per plant) were infiltrated per experiment.

\textit{Plasmid Construction for Bimolecular Fluorescence Complementation (BiFC) and Co-immunoprecipitation (co-IP)—See supplemental Table S1 for a list of all primers that were used to construct and/or mutagenize the DNA clones described below. To construct the binary vector expressing the cYFP-\textit{RTE1} fusion, we first used PCR to simultaneously amplify and fuse the full-length \textit{RTE1} coding sequence downstream of the cYFP sequence, which encodes the C-terminal portion of YFP (amino acids 156–239), using an \textit{RTE1} cDNA clone and the \textit{pSYCE-35S/pUC-SPYCE} vector (Walter et al., Ref. 33) as respective templates. The cYFP-\textit{RTE1} gene fusion fragment was cloned into the Gateway entry vector pDONR221 using the Gateway recombination system (Invitrogen). The cYFP-\textit{RTE1} gene fusion in pDONR221 was verified by DNA sequencing and then transferred into the Gateway binary vector \textit{pH2GW7} (32), which contains the CaMV 35S promoter, to produce \textit{pH2GW7-cYFP-RTE1}. For the \textit{etr1-1} mutant version encoding the C161Y substitution, the corresponding G-to-A mutation was introduced into the above \textit{pH2GW7-cYFP-RTE1} construct by site-directed \textit{in vitro} mutagenesis using the QuikChange kit (Stratagene) to produce \textit{pH2GW7-cYFP-etr1-1}. For \textit{RTH}, the binary vector expressing the cYFP-RTH fusion was constructed in the same way as cYFP-\textit{RTE1}, using the \textit{RTH} coding sequence in place of \textit{RTE1}.

To generate the constructs encoding the \textit{ETR1-nYFP} and \textit{ETR1(1–349)-nYFP} fusions, the coding sequences for full-length \textit{ETR1} and truncated \textit{ETR1} (encoding amino acids 1–349) were each PCR-amplified from an existing ETR1 cDNA template (16), cloned into pDONR221, verified by DNA sequencing, and then transferred into the binary vector \textit{pSPYNE-35S-GW} (33) using the Gateway system. The \textit{etr1-1} and \textit{etr1-2} full-length coding sequences were cloned into \textit{pSPYNE-35S-GW} by the same strategy from existing cDNA clone templates.\textsuperscript{4} Gene fusions of \textit{ECA1-nYFP} and \textit{ERS1-nYFP} in \textit{pSPYNE-35S-GW} were cloned in the same way as \textit{ETR1-nYFP}, using \textit{ERS1} and \textit{ECA1} coding sequences, respectively, in place of \textit{ETR1}.

To create the construct encoding nYFP-ETR1, we first cloned the \textit{ETR1} coding sequence into \textit{pSPYNE-35S/pUC-SPYCE} (33) using restriction enzymes Ascl and Clal. Since \textit{pSPYNE-35S/pUC-SPYCE} carries nYFP downstream of the
cloning site, and we wanted an N-terminal fusion, we removed the C-terminal nYFP from the resulting construct using restriction enzymes XmaI and SstI. Next we PCR-amplified nYFP using pSPYNE-3SS/pUC-SPYNE as a template and then cloned the amplified nYFP fragment using XbaI and Ascl at the N terminus of ETR1 within the altered pSPYNE-3SS/pUC-SPYNE construct. The fusion was confirmed by DNA sequencing. The nYFP-ETR1 gene fusion was then PCR-amplified and cloned into pDONR221 and subsequently transferred into pH2GW7 using the Gateway system.

To generate the construct encoding HA-RTE1 for co-IP, the full-length RTE1 coding sequence was first cloned into pDONR221, verified by DNA sequencing, and then cloned between the CaMV 3SS promoter and hemagglutinin epitope (HA) tag in the binary vector pEarleyGate201 (34) using the Gateway system.

Fluorescence Microscopy—Imaging of YFP fluorescence in tobacco leaf or Arabidopsis seedlings was conducted under a laser scanning confocal microscope (Zeiss LSM510). The excitation wavelength used for YFP was 488 nm, and the emission filter wavelength was 520–550 nm. For visualization, tobacco leaf pieces or cotyledons and root fragments of Arabidopsis seedlings were directly mounted on glass slides in a drop of water. For each experiment, at least ten different samples were examined under the laser scanning microscope. Experiments were repeated three times.

Co-immunoprecipitation (co-IP)—To check the expression levels of the two constructs used for co-IP, we isolated the membrane fraction of Arabidopsis and carried out SDS-PAGE and Western blotting as previously described (16). For co-IP, 3-day-old dark-grown seedlings were homogenized in extraction buffer (250 mM sucrose, 25 mM Hepes-KOH, pH 7.5, 10 mM MgCl₂, 1 mM DTT, 1% Triton X-100) with protease inhibitors. The homogenate was sonicated (20 times) at 6 watts using a Model 100 Sonic Dismembrator (Fisher Scientific) and incubated at 4 °C for 3 h. The homogenate was then centrifuged at 2451 g for 10 min to remove debris. The protein extract was incubated with anti-HA monoclonal antibody (Roche Applied Science) in 2× IP buffer (25 mM HEPES-KOH, pH 7.5, 100 mM NaCl, 0.5 mM EDTA, 3 mM MgCl₂) at 4 °C for 4 h followed by additional incubation at 4 °C for 6 h with protein A-Sepharose beads. The beads were washed three times in IP buffer with 1% Triton X-100. Protein was eluted from the beads and separated by 10% SDS-PAGE followed by immunoblotting using anti-c-Myc (A-14) polyclonal (Santa Cruz Biotechnology or Roche Applied Science), anti-HA monoclonal, anti-SYP21 or anti-SYP61 antibodies (35, 36). To demonstrate that the extract used for co-IP was free of non-solubilized microsome vesicles (i.e. contained fully solubilized protein), similarly prepared extracts were subjected to ultracentrifugation, and then the pellet and soluble fractions were analyzed by immunoblotting (supplemental data Fig. S1).

Cloning, Expression, and Purification of Arabidopsis ETR1, RTE1, and RTE1-1 in E. coli—For ETR1, we used the existing clone pET16b-ETR1, which encodes a tryptophan-less ETR1 (with phenylalanine or leucine substituting for the endogenous tryptophan residues at positions 11, 53, 74, 182, 265, 288, and 563) (37, 38). The tryptophan-less ETR1 was expressed in Escherichia coli and purified as previously described (37, 38). To clone the full-length RTE1 coding sequence into expression vector pET15b (Novagen), PCR was used to amplify the RTE1 coding sequence with flanking Ndel and BamHI restriction sites using pDONR221-ETR1 as the template. The amplified fragment was then cloned into the pET15b vector at the Ndel and BamHI cloning sites and verified by sequencing. The RTE1-1 mutant version was created by in vitro site-directed mutagenesis of the wild-type RTE1 template in pET15b. Using PCR, a fragment was amplified using an rte1-1 mutagenesis primer as the forward primer together with a reverse primer that anneals to the vector. The PCR product was then used as a reverse primer with a forward primer using pET15b-RTE1 as template. The final product was cleaved with Ndel and BamHI and ligated into the pET15b vector digested with Ndel and BamHI. The clones were verified by nucleotide sequencing. The primer sequences used for cloning and mutagenesis are shown in supplemental Table S1. The resulting plasmids pET15b-RTE1 and pET15b-RTE1-1 each containing an N-terminal hexahistidine tag were transformed into E. coli strain BL21 Gold (DE3). Cultures were grown at 30 °C and expression of RTE1 or RTE1-1 was induced by the addition of 0.3 mM IPTG (isopropyl β-d-thiogalactoside) at an optical density of 0.7. Cells were harvested 4 h after induction by centrifugation and stored at −70 °C. The cell pellet was resuspended in 30 mM Tris-sulfate, pH 7.5, 200 mM NaCl, 0.002% (w/v) PMSF (phenylmethylsulfonyl fluoride) and passed through a French pressure cell at 12,000 psi. After centrifugation at 100,000 × g for 60 min the pellet was resuspended in the same buffer. RTE1 (or RTE1-1) was solubilized at room temperature by the addition of 1% (w/v) FOS-CHOLINE®-15 (Anatrace). Unsolubilized material was removed by centrifugation at 100,000 × g. The supernatant was applied to a Ni-IDA (nickel-iminodiacetic acid) affinity column (0.5 cm diameter × 7 cm length) previously equilibrated with 30 mM Tris-Sulfate, pH 7.5, 200 mM NaCl, 0.002% (w/v) PMSF, and 0.05% (w/v) FOS-CHOLINE®-15. The column was washed with 30 bed volumes of the same buffer containing increasing concentrations of imidazole (25–100 mM) to remove contaminating proteins. Bound RTE1 or RTE1-1 was eluted with 10 column volumes of the same buffer containing 250 mM imidazole. The imidazole concentration of the purified RTE1 (or RTE1-1) was decreased to 25 mM by adding 30 mM Tris-sulfate, pH 7.5, 200 mM NaCl, and 0.05% (w/v) FOS-CHOLINE®-15. Purification of recombinant ETR1 and RTE1 was examined by SDS-PAGE on 12 or 15% polyacrylamide gels (39) and visualized by silver staining (40) (supplemental data Fig. S2).

Tryptophan Fluorescence Spectroscopy—Quenching of steady-state tryptophan fluorescence was measured with a LS-55 Luminescence spectrophotometer (Perkin Elmer) at an excitation wavelength of 295 nm. Measurements were carried out with 0.2 μM of the purified recombinant RTE1 at 20 °C in a Quartz SUPRASIL macro/semi-micro cuvette (Perkin Elmer) containing 30 mM Tris-HCl, pH 7.5, 180 mM NaCl, 10 mM KCl, 0.05% (w/v) FOS-CHOLINE®-15, 0.1% (w/v) β-d-dodecylmaltoside, and 0.002% (w/v) PMSF. Purified trypto-
C-terminal fusion to nYFP, since genetic evidence has suggested that RTE1 acts through ETR1 residues 1–349 (28). Fluorescence was still observed, although it was weaker than that seen with full-length ETR1 (Fig. 1B and Table 1). To examine the specificity of the cYFP-RTE1 and ETR1-nYFP association, we assayed BiFC using another ER membrane-localized protein, ECA1 (a Ca^{2+}-ATPase, 44), in place of ETR1 ethylene receptor has three transmembrane domains and has nYFP (amino acids 1–154) fused to the ETR1 C terminus in the cytoplasm (43). Arabidopsis RTE1 has two to four predicted transmembrane domains (25) (two transmembrane domains are depicted) and has cYFP (aa 155–238) fused to the RTE1 N terminus. The cytoplasmic location of the RTE1 N terminus is deduced from the positive signal in 8. The location of the RTE1 C terminus has not been determined. B, representative images from confocal laser scanning microscopy show chlorophyll autofluorescence (left) and reconstituted YFP fluorescence (right) in leaf epidermal cells of 3-week-old soil grown tobacco (N. benthamiana) 48 h after co-infiltration with Agrobacterium harboring cYFP-RTE1 and either ETR1-nYFP, ETR1(1–349)-nYFP, or ERS1-nYFP. Scale bar, 50 μm.

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TABLE 1

Summary of BiFC results

BiFC signal intensity is indicated by “+++”, “++”, and “+” for strong to weak intensity, and “0” for no signal detected. Signal intensity was assessed under the confocal laser-scanning microscope. Experiments were carried out in triplicate, with at least ten independent plant samples examined for each replicate.

| cYFP construct | nYFP construct | Tobacco leaf epidermal cells | Arabidopsis root cells | Arabidopsis cotyledon epidermal cells |
|----------------|----------------|-----------------------------|------------------------|-------------------------------------|
| cYFP-RTE1      | ETR1-nYFP      | +++                         | +++                    | ++                                  |
| cYFP-RTE1      | nYFP-ETR1      | 0                           | 0                      | 0                                   |
| cYFP-RTE1      | ETR1(1–349)-nYFP | +                          | +                      | 0                                   |
| cYFP-RTE1      | ECA1-nYFP      | +                           | +                      | +                                   |
| cYFP-RTE1      | ERS1-nYFP      | +                           | +                      | 0                                   |
| cYFP-RTE1      | ETR1(nYFP)     | 0                           | nd²                    | nd                                  |
| cYFP-RTE1      | etr1-1-nYFP    | +                           | +                      | nd                                  |
| cYFP-RTE1      | etr1-2-nYFP    | +                           | nd                     | nd                                  |
| cYFP-RTE1      | etr1-1-nYFP    | +                           | nd                     | nd                                  |

²: nd, not determined.

receptor most closely related to ETR1 (67% overall amino acid identity; 72% amino acid identity for residues 1–349). Using an ERS1 ethylene receptor carrying a C-terminal fusion to nYFP, a weak signal was detected (Fig. 1C and Table 1), suggesting that RTE1 might be capable of associating with ERS1. We also examined whether mutations in ETR1 could have an effect on the interaction with RTE1. We tested two gain-of-function mutations, etr1-1 and etr1-2, which both confer ethylene insensitivity in Arabidopsis; etr1-2 requires RTE1 for ethylene insensitivity, whereas etr1-1 is independent of RTE1 (25). Despite this functional difference, both etr1-1-nYFP and etr1-2-nYFP showed interaction with cYFP-RTE1, although the interaction appeared to be weaker than with wild-type ETR1 (Fig. 2 and Table 1).

Conversely, we assayed the ETR1-nYFP fusion for interaction with the Arabidopsis RTE1 homolog, RTH (25), which has 51% identity to RTE1 over 209 amino acids and localizes to the same subcellular organelles as RTE1.³ Despite the similarities between RTE1 and RTH, co-infiltration of cYFP-RTH and ETR1-nYFP gave no BiFC signal (Table 1 and data not shown), suggesting that the signal produced by ETR1-nYFP paired with cYFP-RTE1 might be specific to RTE1.

BiFC of RTE1 and ETR1 in Stably Transformed Arabidopsis—The association of RTE1 and ETR1 was also visualized in Arabidopsis. Arabidopsis was stably transformed with each of the following binary constructs, all driven by the CaMV 35S promoter: cYFP-RTE1, ETR1-nYFP, ETR1(1–349)-nYFP, ERS1-nYFP, ECA1-nYFP, and nYFP-ETR1. Homozygous lines were generated for each transgene, and then genetically crossed to produce F1 progeny. BiFC was examined in the epidermal cells of roots and cotyledons of 1-week-old F1 seedlings. Consistent with the tobacco epidermal cell results, transgenic F1 progeny harboring both cYFP-RTE1 and ETR1-nYFP produced detectable BiFC signals in both root and cotyledon epidermal cells (Fig. 3 and Table 1), while a weak signal was detected only in root cells (with no signal in cotyledon cells) when cYFP-RTE1 was paired with either ETR1(1–349)-nYFP or ERS1-nYFP (Table 1 and data not shown). Unfortunately we were unable to detect the various fusion proteins when we carried out Western blotting to assess their expression levels (data not shown). Thus it is important to note that the BiFC signal strength does not necessarily correlate with protein–protein interaction strength.

Co-IP of RTE1 and ETR1 in Stably Transformed Arabidopsis—To confirm the association of RTE1 and ETR1, we carried out co-IP using epitope-tagged versions of RTE1 and ETR1 expressed in stably transformed Arabidopsis. RTE1 was tagged at its N terminus with an HA epitope. A homozygous transgenic line expressing HA-RTE1 (driven by the
Co-IP of ETR1 and ETR1. Protein extracts of 3-day-old dark-grown F1 seedlings from genetic crosses of stably transformed Arabidopsis carrying ETR1–5xMyc and HA–RTE1, respectively, were subjected to immuno-precipitation using an anti-HA antibody (+HA). Total protein extracts (10%) (T), the third wash (W3) and the immunoprecipitates (IP) were separated by SDS-PAGE followed by immunoblotting using anti-c-Myc and anti-HA antibodies to detect ETR1–5xMyc and HA–RTE1, respectively. As a negative control, anti-SYP61 and anti-SYP21 antibodies were used to detect SYP61 (36) and SYP21 (35), which are transmembrane proteins localized to the trans-Golgi network and prevacuolar compartment, respectively. As an additional control, immunoprecipitation was performed in the absence of the primary anti-HA antibody (−HA).

CaMV 35S promoter) was crossed with a previously generated transgenic line expressing a functional ETR1–5xMyc fusion under the control of the native ETR1 promoter (16). The resulting F1 progeny expressing both HA–RTE1 and ETR1–5xMyc were used for co-IP. Total protein extracts were first incubated with or without anti-HA antibody, followed by precipitation with protein A-Sepharose. The immunoprecipitated fraction was run on a polyacrylamide gel and immunoblotting using an anti-c-Myc antibody showed the presence of ETR1–5xMyc only when the anti-HA antibody was used (Fig. 4). In contrast, the anti-HA antibody did not result in IP of SYP61 (a SNARE protein) (36) or SYP21 (synaptin) (35), which are transmembrane proteins localized to the trans-Golgi network and prevacuolar compartment, respectively, thus demonstrating the specificity of the anti-HA co-IP. Co-IP of ETR1-5xMyc was detected in three independent experiments, thus confirming the in vivo protein association of RTE1 and ETR1. Similar results were obtained for the reciprocal experiment, using anti-c-Myc for the co-IP (data not shown); however the anti-c-Myc antibody gave nonspecific background in Western blotting, so the specificity of the IP could not be ensured.

**Quantitative Analysis of the RTE1–ETR1 Interaction by Tryptophan Fluorescence Spectroscopy**—We next tested whether the interaction of RTE1 and ETR1 could occur in the absence of other proteins. The interaction and stability of the RTE1–ETR1 binary complex was examined in vitro using tryptophan fluorescence quenching. Full-length RTE1 (supplemental Fig. S2) and ETR1 proteins were expressed in and purified from E. coli. Correct folding and structure of the purified recombinant RTE1 was indicated by circular dichroism (supplemental Fig. S3). For ETR1, we used an existing functional tryptophan-less version of ETR1, in which seven tryptophan residues were replaced with either phenylalanine or leucine residues (38). The tryptophan-less version was necessary to allow for the titration measurements of tryptophan fluorescence in the RTE1 protein. Titration of the purified RTE1 with the tryptophan-less ETR1 protein yielded an apparent \( K_d \) of \( \sim 117 \) nM (Fig. 5). This relatively low dissociation constant provided evidence of a high affinity interaction between RTE1 and ETR1.

**Reduced Affinity of the RTE1–ETR1 Interaction Caused by the rte1-1 Mutation**—The Arabidopsis rte1-1 mutation, encoding a C161Y substitution, was previously isolated by genetic screening and confers an etr1 loss-of-function phenotype (25). We tested whether the C161Y substitution has an effect on the molecular association of RTE1 and ETR1 using BiFC and tryptophan fluorescence spectroscopy. For BiFC, the rte1-1 mutation was introduced into the cYFP–RTE1 construct using in vitro site-directed mutagenesis. The resulting cYFP–rte1-1 mutant version was assayed for BiFC with ETR1–nYFP in tobacco leaf epidermal cells. In contrast to the positive signal obtained for wild-type cYFP–RTE1 and ETR1–nYFP, no signal was detected for cYFP–RTE1-1 and ETR1–nYFP, suggesting that the rte1-1 mutation reduces the RTE1–ETR1 interaction (Table 1 and data not shown).

To test the effect of the rte1-1 mutation on the ETR1–RTE1 complex using tryptophan fluorescence spectroscopy, the full-length RTE1 clone was mutagenized to encode the rte1-1 (C161Y) substitution, and the resulting RTE1-1 product was expressed in and purified from E. coli (supplemental Fig. S2). Based on circular dichroism, there were no substantial secondary structure changes between the purified recombinant RTE1-1 mutant protein and the purified recombinant RTE1 wild-type protein (supplemental Fig. S2). Titration of the purified recombinant RTE1-1 mutant protein with the tryptophan-less ETR1 protein showed a nearly 12-fold increase in the dissociation constant of the ETR1–RTE1-1 complex (1.38 \( \mu M \)) compared with titration of the purified recombinant wild-type RTE1 (Fig. 5). This increase indicates a substantial...
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decrease in the interaction of both binding partners due to the single mutation in RTE1-1 (C161Y).

**DISCUSSION**

RTE1 is a novel protein conserved in plants and animals. In Arabidopsis, RTE1 negatively regulates ethylene signaling (25), but the molecular mechanism of this regulation is unknown. RTE1 and ETR1 are both transmembrane proteins that co-localize to the ER and Golgi apparatus (16). Genetic data suggest that RTE1 promotes the signaling state of the ETR1 ethylene receptor (26). Here, we demonstrate a physical association between RTE1 and ETR1 in vivo and in vitro. Our findings suggest that RTE1 action in ethylene signaling involves a direct interaction with ETR1.

We first detected association of RTE1 and ETR1 using BiFC in a transient assay in tobacco leaf epidermal cells. Subsequently, we detected the interaction in stably transformed Arabidopsis using both BiFC and co-IP. The BiFC signal was observed when the N terminus of RTE1 and the C terminus of ETR1 were fused to cYFP and nYFP, respectively. We had previously shown that fusing RFP and a 5xMyc epitope tag at these particular termini of RTE1 and ETR1, respectively, does not disrupt RTE1 or ETR1 function in Arabidopsis (16). The C terminus of ETR1 is very likely to be in the cytoplasm based on the topology of a melon ethylene receptor (43). RTE1 is predicted to have two to four transmembrane domains (25) but the membrane topology of RTE1 is unknown. We found that RTE1 lacks N-linked glycosylation, precluding the use of N-linked glycosylation to determine the topology. Nevertheless, the positive BiFC signal produced by the combination of cYFP-RTE1 and ETR1-nYFP suggested that the N terminus of RTE1 lies on the cytoplasmic side of the membrane, which is where the C terminus of ETR1 is believed to reside. Moreover, the subcellular pattern of the BiFC signal was comparable to that of GFP-tagged ETR1. In addition, we obtained a BiFC signal for cYFP-RTE1 paired with ETR1(1–349)-nYFP. ETR1(1–349) has been shown to have the same subcellular localization as wild-type ETR1 (14). This is consistent with genetic data that indicates RTE1 promotes ETR1 signaling via the ETR1 N terminus (residues 1–349) (28).

The in vivo molecular interaction of RTE1 and ETR1 was confirmed by co-IP in Arabidopsis, and the affinity of the interaction was examined by tryptophan fluorescence quenching measurements, using purified RTE1 and ETR1 proteins expressed in E. coli. Association in vitro occurred with high affinity, based on the determined $K_d$ value of $\sim 117$ nM. This low $K_d$ value is indicative of a specific interaction, rather than a nonspecific interaction arising from hydrophobic interactions, and is comparable to that of other known, highly specific protein-protein interactions, such as NusB:S10 (46), the NusA:core RNA polymerase (47), Ras:Raf (48), and EGF:EGF receptor (49). This in vitro association suggests that the physical interaction of RTE1 and ETR1 does not require the presence of other plant proteins.

The rte1-1 mutation encodes a C161Y substitution and confers an ethylene hypersensitive phenotype similar to the etr1 loss-of-function phenotype. Significantly, we found that the rte1-1 mutation reduces the affinity of the molecular association of RTE1 with ETR1, in both BiFC and fluorescence spectroscopy. The dissociation constant for the in vitro association was nearly 12-fold higher for the recombinant RTE1-1 mutant protein compared with the recombinant ETR1 wild-type protein. Thus, the physical interaction of ETR1 with RTE1 appears to be required to maintain ETR1 function.

BiFC signals were observed for wild-type RTE1 and the two dominant gain-of-function mutants, etr1-1 and etr1-2, even though etr1-1 signaling is independent of RTE1. This suggests that the RTE1 independence of the etr1-1 allele is not due to a loss of association between ETR1-1 and RTE1. Perhaps the particular conformation of ETR1-1, or an interacting protein, is the basis for its RTE1 independence.

Although genetic analysis indicates that RTE1 specifically acts on ETR1 and not on the other Arabidopsis ethylene receptors (25, 27, 28), we also detected a weak signal for cYFP-RTE1 paired with ERS1-nYFP, which suggests that RTE1 might interact to some extent with the ERS1 receptor. Given that ERS1 and ETR1 are likely to be in contact with each other in the ethylene receptor complex (23), it is conceivable that there was reconstitution of the BiFC signal based on close proximity of the YFP halves, or that there is actually a nonfunctional interaction of RTE1 and ERS1. The absence of detectable interaction between cYFP-ERT1 homolog) and ETR1-nYFP is consistent with data suggesting that the RTE1 homolog, RTH, does not play the same role as RTE1 in ethylene signaling.

The physical association of RTE1 and ETR1 is consistent with the highly specific functional interaction of RTE1 and ETR1 that has been suggested by genetic analyses. RTE1 and ETR1 have possibly co-evolved, as implicated by their physical and functional association. This is interesting in light of the fact that RTE1 is present not only in plants, but is conserved in metazoans and some fungi. Because these other organisms are not known to encode ethylene receptors, RTE1 homologs might possess a more general conserved cellular function.

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