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Ethylene Receptor 1 (ETR1) Is Sufficient and Has the Predominant Role in Mediating Inhibition of Ethylene Responses by Silver in *Arabidopsis thaliana*[*] [S]

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Background: Silver ions block ethylene perception yet support ethylene binding to Ethylene Receptor 1 (ETR1).
Results: Loss of ETR1 reduces the effects of silver while loss of the other receptors has less of an effect.
Conclusion: ETR1 has the predominant role and is sufficient for the effects of silver.
Significance: This could underlie differences in the roles of the receptors in plants.

Ethylene influences many processes in *Arabidopsis thaliana* through the action of five receptor isoforms. All five isoforms use copper as a cofactor for binding ethylene. Previous research showed that silver can substitute for copper as a cofactor for ethylene binding activity in the ETR1 ethylene receptor yet also inhibit ethylene responses in plants. End-point and rapid kinetic analyses of dark-grown seedling growth revealed that the effects of silver are mostly dependent upon ETR1, and ETR1 alone is sufficient for the effects of silver. Ethylene responses in *etr1-6 etr2-3 ein4-4* triple mutants were not blocked by silver. Transformation of these triple mutants with cDNA for each receptor isoform under the promoter control of *ETR1* revealed that the *cETR1* transgene completely rescued responses to silver while the *cETR2* transgene failed to rescue these responses. The other three isoforms partially rescued responses to silver. Ethylene binding assays on the binding domains of the five receptor isoforms expressed in yeast showed that silver supports ethylene binding to ETR1 and ERS1 but not the other isoforms. Thus, silver may have an effect on ethylene signaling outside of the ethylene binding pocket of the receptors. Ethylene binding to ETR1 with silver was ~30% of binding with copper. However, alterations in the K_d for ethylene binding to ETR1 and the half-time of ethylene dissociation from ETR1 do not underlie this lower binding. Thus, it is likely that the lower ethylene binding activity of ETR1 with silver is due to fewer ethylene binding sites generated with silver *versus* copper.

Ethylene is a gaseous plant hormone that influences a number of processes in higher plants such as seed germination, abscission, senescence, fruit ripening, response to stress, and growth. In etiolated *Arabidopsis thaliana* seedlings, ethylene causes a number of changes including reduced growth of the hypocotyl and root, increased radial expansion of the hypocotyl, increased tightening of the apical hook, and an increase in root hair formation (1). Responses to ethylene are mediated by a family of five receptors in *Arabidopsis* (2–5). Based upon domain structure and sequence comparisons of the ethylene binding domain, the ethylene receptors in *Arabidopsis* can be divided into two subfamilies (Fig. 1) (6). Subfamily I consists of ETR1[2] (ethylene receptor 1) and ERS1 (ethylene response sensor 1) and subfamily II includes ETR2, ERS2, and EIN4 (ethylene insensitive 4) (2–5).

All five receptor isoforms are involved in ethylene signaling and have overlapping roles that regulate various phenotypes such as growth (2, 4, 6–8). However, it is also clear that the five receptor isoforms in *Arabidopsis* are not entirely redundant in their roles (9–21). This appears to be a general feature of ethylene signaling since only specific receptor isoforms mediate fruit ripening in tomato (22). The basis for these non-overlapping roles is unclear but may involve structural or functional differences. The ethylene receptors are homologous to two-component receptors and have three membrane-spanning α-helices at the N-terminal region containing the ethylene-binding domain followed by a GAF domain and a domain with similarities to bacterial histidine kinases (Fig. 1). The subfamily II receptors have an extra hydrophobic region at the N terminus that might function as a signal sequence. Two-component receptors transduce signals via His autophosphorylation followed by the transfer of that phosphate to an Asp residue in the receiver domain (23). However, not all the ethylene receptor isoforms have His kinase activity (24, 25). Additionally, only three of the five receptor isoforms (ETR1, ETR2, EIN4) contain a receiver domain at the C terminus (Fig. 1). Alternatively, the non-overlapping roles of the receptors may be due to other proteins that modulate specific receptor isoforms. For instance, RTE1 (reversion to ethylene sensitivity 1) is a protein that has recently been shown to specifically interact with and affect ETR1 (26–29). This modulation may occur through interactions with the ETR1 ethylene binding domain (30, 31).

It has been shown that copper is required for high-affinity ethylene binding in exogenously expressed ETR1 receptors (32) supporting earlier speculations about the requirement for a

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[2] The abbreviations used are: ETR1, ethylene receptor 1; GAF, cGMP phosphodiesterase/adenyl cyclase/FhlA; 1-MCP, 1-methylcyclopropene.

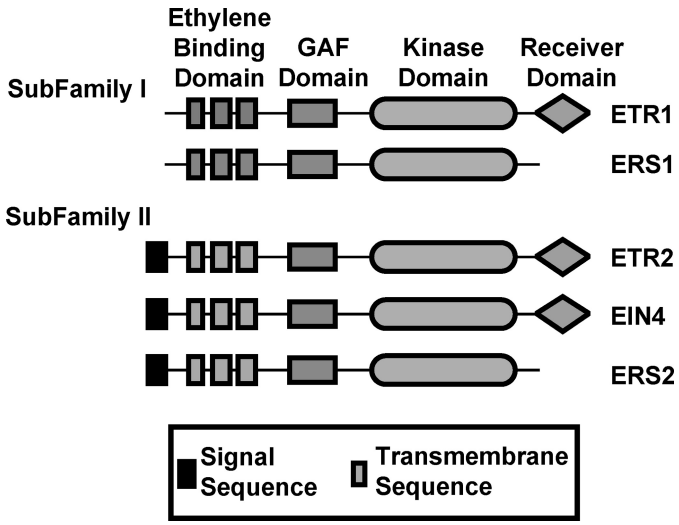

FIGURE 1. **Domains of the ethylene receptors from *Arabidopsis*.** All of the receptor isoforms contain ethylene binding, GAF and kinase domains. A subset of the receptors contain a receiver domain and subfamily II receptors have an extra N-terminal sequence as shown.

transition metal cofactor for ethylene binding (33–36). This requirement for copper is likely to be a general feature of all ethylene receptors in plants (15). Additionally, prior studies indicate that RAN1 (response to antagonist 1) is a copper transporter that acts upstream of the receptors and is required for normal biogenesis of the receptors (37–40). Interestingly, the *etr1-1* mutant protein fails to coordinate copper and is unable to bind ethylene (32, 41). Together, these studies have led to a model where copper ions are delivered to and required by the ethylene receptors for ethylene binding. It is thought that ethylene binding causes a change in the coordination chemistry of the copper cofactor resulting in a change in the binding site that is transmitted through the receptor to downstream signaling elements (42).

Of many other transition metals previously tested, only the two other Group 11 transition metals (silver and gold ions) supported the binding of ethylene to ETR1 (32, 43). This observation is of interest since silver has long been recognized for its ability to block ethylene responses in plants (34). Since Ag^+ is larger than Cu^+, a model has been developed proposing that silver occupies the binding site and interacts with ethylene but prevents stimulus response coupling through the receptors because of steric effects (19, 32, 43, 44). However, there is some evidence indicating that the action of silver on ethylene responses in *Arabidopsis* is not so clear-cut and may only involve the subfamily I receptors (12, 45). If true, this suggests that the ethylene-binding domains of the subfamily I and II receptors are different from each other. In the current study we examined the ability of silver to block ethylene responses in a variety of receptor-null plants using both end-point and growth kinetic analyses. This information was compared with the ability of silver to act as a cofactor for ethylene binding to exogenously expressed ethylene receptors. We also further characterized the effects of silver on the ETR1 receptor. Results presented in the current study support more complex models for the effects of silver on ethylene receptor function.

EXPERIMENTAL PROCEDURES

The *etr1-6*, *etr1-7*, *etr2-3*, *ers2-3*, and *ein4-4* mutants were originally obtained from Elliot Meyerowitz (3), the *ers1-3* and *etr1-9* mutants were from Eric Schaller (11), the *ers1-3;etr2-3; ein4-4;ers2–3* quadruple mutants were obtained from Chi-Kuang Wen (9, 20), and the *rte1-2* mutants were from Caren Chang (26). The *etr1-6*, *etr1-7*, and *etr1-9* loss-of-function mutants have previously been shown to be similar since they result in similar alterations in phenotypes (3, 11). Other combinatorial mutants used in this study have previously been described (10, 11, 46). All mutants are in the Columbia (Col) background except for *etr1-9*, *ers1-3*, and *ers1-2* that are in the Wassileweskija (Ws) background. All transgene constructs and transgenic plant lines have been described previously (13, 16, 20, 46). $^{14}C_2H_4$ was obtained from American Radiolabeled Chemicals (St. Louis, MO).

Seed Preparation, Growth Measurements, and Imaging—*Arabidopsis thaliana* seeds were surface sterilized and germinated as previously described (16, 43, 47). For silver treatment, 100 μM silver nitrate was included in the agar. End-point analysis growth experiments using 10 seeds per condition were carried out as previously described (43) except that 100 μl/liter ethylene was used, and the gas flow rate was maintained at 50 ml min^{-1}. Growth kinetic experiments were carried out, analyzed, and normalized to the growth rate in air prior to ethylene treatment as previously described (16, 47–49). All growth experiments were carried out in the dark. Infrared light emitting diodes were used for imaging during growth kinetic experiments. Images of unfixed seedlings grown and treated as described above were acquired using a CanoScan 4400F flatbed digital scanner (Canon, Lake Success, New York).

Ethylene Concentration Measurements—Ethylene concentrations were determined using a Hewlett-Packard 6890 gas chromatograph with an HP Plot/Q column (Agilent Technologies, Santa Clara, CA) or an ETD-300 photoacoustic laser spectrophotometer (Sensor Sense, The Netherlands).

DNA Constructs, Cell Strains, Growth Conditions, and Membrane Isolation—Pichia pastoris (Invitrogen) was used to express the binding domain of each receptor fused to GST (glutathione *S*-transferase). We used the following nomenclature for these constructs: ETR1[1–128]-GST, ETR2[1–157]-GST, ERS1[1–128]-GST, ERS2[1–160]-GST, EIN4[1–151]-GST for the binding domains of ETR1, ETR2, ERS1, ERS2, and EIN4 respectively fused to GST (15). These constructs were described and characterized previously using *Saccharomyces cerevisiae* as the expression system (15, 32).

To generate these constructs for use in *P. pastoris*, the sequence encoding the ethylene binding domain of each receptor was amplified by PCR using cDNA generated from Col seedlings. The GST sequence was PCR amplified using the pGEX vector. The receptor-specific primers introduced the EcoRI restriction site at the N terminus and KpnI at the C terminus and the GST-specific primers introduced KpnI at the N terminus and ApaI at the C terminus. The ETR1[1–128] construct was generated by PCR amplification using the forward primer 5′-AATTCATAGCCACCATGGAAGTCTGCAAT-3′ and the reverse primer 5′-ATATAGGTACCCTCAGCAGCTTTAT-

TTTTCA-3′, ERS1[1–128] using the forward primer 5′-AATTCATAGCCACCATGGAGTCATGCGAT-3′ and the reverse primer 5′-CTAATGGTACCCTCATCAGCTTTCTTC-3′, ETR2[1–157] using the forward primer 5′-AATTCATAGCCACCATGGTTAAAGAAATAGCT-3′ and the reverse primer 5′-ACGATAGGTACCCTCATGAGCTTTCTT-3′, ERS2[1–160] using the forward primer 5′-AATTCATAGCCACCATGTTAAAGACATTG-3′ and the reverse primer 5′-CTAATGGTACCCTCTCTGGTCTTCTTAC-3′, and EIN4[1–151] using the forward primer 5′-AATTCATAGCCACCATGTTAAGATCTTTA-3′, and the reverse primer 5′-ATATAGGTACCCTCCAACACATTCTG-3′. The GST sequence was amplified using the forward primer 5′-ATAGGTACCATGTCCCCTATACTAGGT-3′, and the reverse primer 5′-ATAATTGGGCCCTTATCAGTCACGATGCG-3′. Following PCR amplification, each fragment was gel purified, digested using EcoRI and KpnI, ligated into the pPICZ A vector, and subsequently transformed into *Escherichia coli*. Plasmids were isolated from positive colonies, and receptor and GST gene fragments were digested with KpnI and ApaI and ligated together. Plasmids containing the complete receptor-GST construct were sequenced to confirm no errors were present, then linearized, and transformed into *P. pastoris* using electroporation. Yeast cultures expressing each construct were grown under conditions described in the Invitrogen Pichia manual for membrane-bound proteins. Following a 48 h induction, the yeast cells were isolated and membranes purified using previously described methods (50). Membranes were rapidly frozen in liquid nitrogen and stored at −80 °C until used.

Ethylene Binding Assays—Prior to assaying ethylene binding activity, 300 μM of either silver nitrate or $CuSO_4$ was added to the assay buffer. In some cases, neither metal salt was added. Saturable ethylene binding to membranes isolated from yeast expressing the binding domain of each receptor isoform fused to GST was determined using the methods of Sisler (51) as modified by others (32, 50). In some cases empty vector controls were included. The time-course of ethylene dissociation from ETR1[1–128]-GST and the K_d for ethylene binding to ETR1[1–128]-GST were determined according to methods from prior studies (15, 41, 52).

RESULTS

Receptor Requirements for the Ethylene Blocking Effects of Silver Nitrate—Previous observations that *Arabidopsis* plants lacking subfamily II receptors still respond to silver while those lacking subfamily I receptors do not implies that silver acts through the subfamily I receptors (12, 45). To more completely characterize this phenomenon, we conducted a more thorough evaluation of the effects of silver on various single and combinatorial receptor loss-of-function mutant seedlings. We initially examined the effects of 100 μM silver nitrate on seedlings grown for 4 days in the dark in air or treated with 100 μl/liter ethylene (Fig. 2). We observed that like their respective wild-type controls, silver nitrate blocked growth inhibition upon application of ethylene in most single receptor loss-of-function seedlings including *etr2-3* and *ein4-4* in the Col background and *ers1-3* and *ers2-3* mutants in the Ws background. Similarly, silver nitrate blocked ethylene responses in *etr2-3;ein4-4* double mutants and *etr2-3;ein4-4;ers2-3* triple mutants (Fig. 2*A*).

In marked contrast to these observations, *etr1-7* and *etr1-9* mutants had a measurably reduced response to silver nitrate (Fig. 2, *A* and *B*). In other words, silver nitrate only partially blocked growth inhibition upon application of ethylene. Interestingly, this is similar to what we have previously observed in the *ran1-1* and *ran1-2* partial loss-of-function mutants (39). Of the combinatorial receptor loss-of-function mutants examined, only the *etr1-6;etr2-3;ein4-4* triple mutant seedlings had an altered response to silver nitrate (Fig. 2, *A* and *C*). In these mutants, silver nitrate had no measurable effect on the magnitude of growth inhibition caused by ethylene. Thus, mutants containing an *etr1* loss-of-function mutation are less responsive to the ethylene response blocking effects of silver.

These results point to a key role for ETR1 in mediating the effects of silver. To confirm this failure to respond to silver is due to ETR1, we transformed *etr1–6;etr2–3;ein4–4* triple mutants with a genomic *ETR1* transgene (*gETR1*). Consistent with prior research (16, 20), the *gETR1* transgene rescued the reduced growth phenotype of the triple mutant (Fig. 2*C*). This transgene also rescued the silver phenotype so that silver nitrate once again blocked ethylene's effects in these transformants (Fig. 2*C*). One distinguishing characteristic of ETR1 is that it has both His kinase activity and a receiver domain with a conserved aspartate for phosphotransfer (24, 25). Therefore, we transformed this triple mutant with a genomic *ETR1* transgene lacking the conserved aspartate required for phosphotransfer (*getr1*[*D*]) to determine if this was required for the silver phenotype. The *getr1*[*D*] transgene rescued the silver phenotype as well as the *gETR1* transgene (Fig. 2*C*) indicating that phosphotransfer through ETR1 is not required. Another distinguishing characteristic of ETR1 is that it is specifically modulated by RTE1 (26–31). However, the ethylene growth inhibition response in *rte1-2* mutants was blocked by silver nitrate (Fig. 2*A*). Thus, the effects of silver on plants do not require phosphotransfer through ETR1 or a functional RTE1.

The Effects of Silver Nitrate on Ethylene Growth Response Kinetics—To better define the effects of silver, we examined the ethylene growth response kinetics of seedlings in the presence and absence of 100 μM silver nitrate. Our prior studies have shown that there are two phases to ethylene-induced growth inhibition that are genetically distinct (16, 47). The first phase starts ~10 min after the addition of ethylene and reaches a plateau in growth rate ~20 min after the addition of ethylene. This first plateau lasts ~30 min and is followed by a second phase of growth inhibition that lasts ~15 min. This second phase requires the presence of the EIN3 and EIL1 transcription factors and ends ~95 min after the addition of ethylene when a new, lower steady state growth rate is reached (16, 47, 53). At saturating concentrations of ethylene, this second plateau of growth inhibition lasts for as long as ethylene is present (13, 54).

In the absence of silver nitrate, both wild-type and mutant seedlings had growth inhibition kinetics similar to our previous reports (16, 47, 53, 54) including prolonged growth inhibition in the continued presence of ethylene (Fig. 3). Treatment with 100 μM silver nitrate completely blocked long-term responses to ethylene in both Col and Ws wild-type seedlings (Fig. 3, *A*

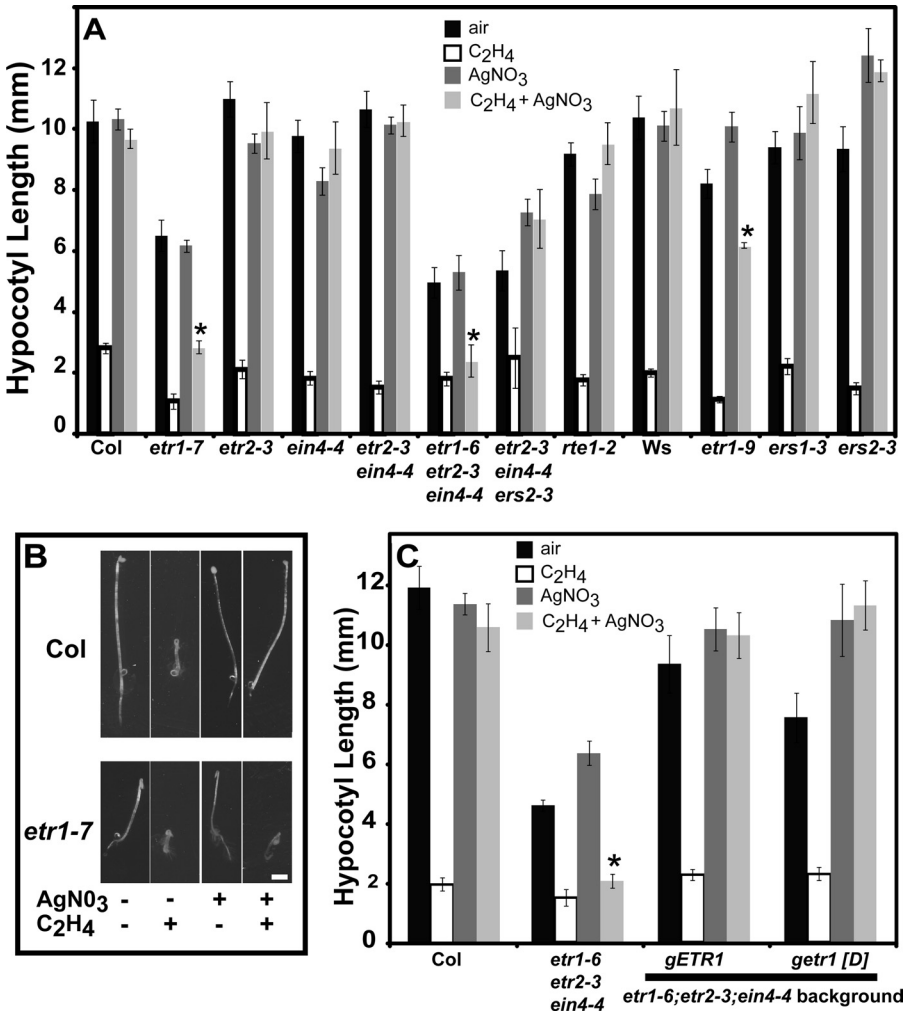

FIGURE 2. **Effect of silver nitrateon ethylene growth responses of dark-grown *Arabidopsis* seedlings.** In all panels, seedlings were grown in darkness for 4 days under the indicated conditions. A concentration of 100 μl/liter ethylene and 100 μM silver nitrate was used. Data represent the mean hypocotyl length ± S.E. Differences between air and ethylene in the presence of silver were analyzed with *t* tests and considered statistically significant with $p < 0.001$ (*). *A*, hypocotyl growth of ethylene receptor loss-of-function and *rte1* mutant seedlings were examined. Wild-type seedlings were included as controls. The *etr1-7*, *etr2-3*, *ein4-4*, *etr2-3;ein4-4*, *etr1-6;etr2-3;ein4-4*, and *etr2-3;ein4-4;ers2-3*, and *rte1-2* mutants are in the Col background while *etr1-9*, *ers1-3*, and *ers2-3* are in the Ws background. *B*, Col and *etr1-7* seedlings treated with air or ethylene in the presence or absence of silver as labeled. Scale bar equals 2 mm. *C*, growth of *etr1-6;etr2-3;ein4-4* triple mutants transformed with a wild-type genomic *ETR1* (*gETR1*) transgene or a mutant transgene that lacks the conserved aspartate in the receiver domain for phosphotransfer (*getr1* [*D*]). *Col*, Columbia; *Ws*, Wassilewskija.

and *B*). However, while all responses to ethylene in Ws were blocked by this concentration of silver nitrate (Fig. 3*B*), Col seedlings still had a very transient response to ethylene (Fig. 3*A*). Thus, the first phase of growth inhibition may be less sensitive to the antagonistic effects of silver. This is consistent with our prior data showing that the first phase of growth inhibition is much less sensitive to 1-MCP (47), a competitive inhibitor of ethylene (55–58).

We also evaluated the effects of silver nitrate on the ethylene growth response kinetics of single loss-of-function receptor mutants. Strikingly, silver nitrate treatment had no obvious effect on the initial growth inhibition kinetics and only a slight effect on the second phase of growth inhibition of the *etr1-7* mutants (Fig. 3*C*). However, in the continued presence of ethylene, the growth rate of *etr1-7* mutants in the presence of silver nitrate started to increase ~2.5 h after ethylene was introduced. The other single loss-of-function receptor mutants had less severe alterations in their responses to silver nitrate (Fig. 3, *D–G*). For *ers1-3*, *etr2-3*, and *ein4-4* (Fig. 3, *D*, *E*, *G*) this was characterized by an attenuated first phase growth inhibition response followed by an increase in growth rate to air pre-treatment levels. Long-term responses to silver nitrate were unaffected in these single loss-of-function mutants. The *ers2-3* mutants showed no response to ethylene in the presence of silver nitrate (Fig. 3*F*).

We also examined the ethylene growth response kinetics of several combinatorial receptor loss-of-function mutants. Application of silver nitrate completely blocked the effects of ethylene on the *etr2-3;ein4-4;ers2-3* triple mutants (Fig. 3*H*). By contrast, the *etr1-6;etr2-3;ein4-4* triple mutants were unaffected by silver nitrate and exhibited no reversal in growth inhibition (Fig. 3*I*). This is in agreement with our end-point analyses above (Fig. 2). These differences are not due to alterations in overall receptor levels since both triple mutant backgrounds have comparable levels of ethylene receptor gene expression and ethylene binding (15). Application of silver nitrate to *ers1-*

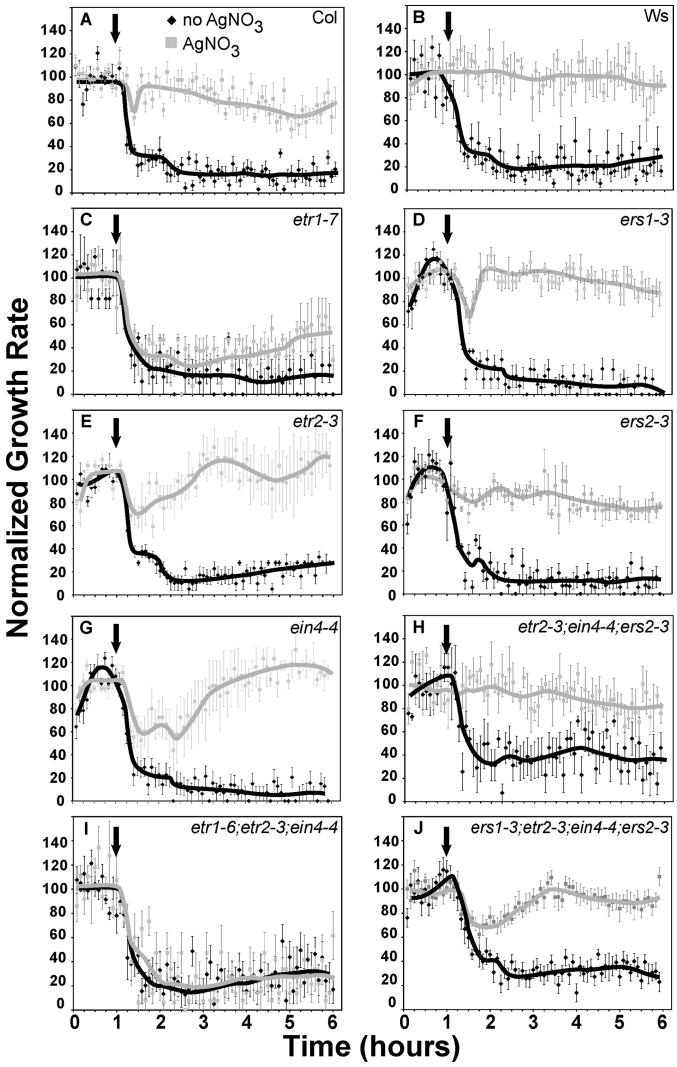

FIGURE 3. **The effect of silver nitrate on ethylene growth response kinetics.** Each panel (*A–J*) shows data for one seed line as designated. Seedlings were grown in air for 1 h followed by application of 1 μl/liter ethylene (*arrow*) for 5 h. The hypocotyl response kinetics of seedlings grown on 100 μM silver nitrate are compared with seedlings grown in the absence of added silver nitrate. Data represent the mean ± S.E. from at least 5 seedlings total from at least four separate experiments. *Lines* were drawn by hand. *Col*, Columbia; *Ws*, Wassilewskija.

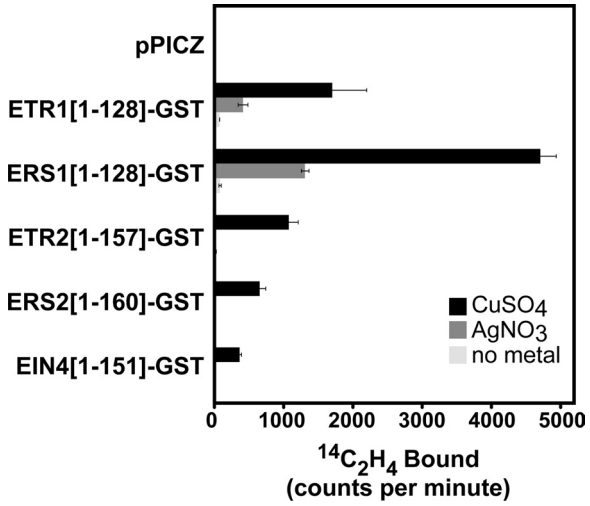

FIGURE 4. **The effects of copper sulfate and silver nitrate on ethylene binding activity in members of the ethylene receptor family from *Arabidopsis*.** Ethylene binding to equal amounts of yeast membranes isolated from yeast cells expressing the binding domain of each receptor isoform fused to GST or empty vector was compared between samples treated with $^{14}C_2H_4$ (0.1 μl/liter) and identical samples treated with $^{14}C_2H_4$ (0.1 μl/liter) plus $^{12}C_2H_4$ (1000 μl/liter). Samples were pre-incubated for 30 min with either 300 μM copper sulfate, silver nitrate or no metal. Displaceable ethylene binding was determined by subtracting the amount of $^{14}C_2H_4$ bound in the presence of excess $^{12}C_2H_4$ from the amount of $^{14}C_2H_4$ in the absence of added $^{12}C_2H_4$. Data show the mean counts per minute ± S.D.

3;etr2-3;ein4-4;ers2-3 quadruple mutants that only contain ETR1 resulted in attenuated first phase responses (Fig. 3*J*) much like that observed with the *etr2-3* and *ein4-4* single mutants.

Together these data indicate that silver nitrate exerts its effects predominantly through ETR1, but that the other isoforms are also involved. Results with the *ers1-3;etr2-3;ein4-4;ers2-3* quadruple mutant seedlings show that ETR1 is sufficient to support the inhibitory effects of silver nitrate on long-term ethylene growth responses.

Silver Nitrate Supports Ethylene Binding to Subfamily I Receptors but Not Subfamily II Receptors—Previously we noted that silver can substitute for copper as a cofactor for ethylene binding in the ETR1 receptor (32, 43). This suggests that silver is blocking ethylene signaling through ETR1 by uncoupling the binding event from receptor output. The question remains, why does ETR1 have such a large role in mediating the effects of silver while the other four receptor isoforms play little or no role? One possibility is that silver binds poorly to the other four receptor isoforms and thus has little or no effect on their functionality. A second possibility is that silver does bind to these receptor isoforms, but does not affect stimulus-response coupling through these receptors. To indirectly determine whether or not silver binds to the binding domain of each receptor isoform, we compared the ethylene binding activity of yeast membranes isolated from yeast expressing empty vector or the ethylene-binding domain of each receptor isoform fused to GST. Membranes were incubated with 300 μM $CuSO_4$, 300 μM $AgNO_3$ or no added metal. We have previously shown that in the presence $CuSO_4$ the binding domain of each receptor isoform binds ethylene at levels proportional to receptor expression levels (15). Consistent with these prior results, all five receptor isoforms retained ethylene binding activity with $CuSO_4$ while membranes isolated from yeast expressing the empty pPICZ vector had no detectable ethylene binding above background (Fig. 4, supplemental Table S1). Also consistent with our prior observations (32, 43), silver nitrate supported ethylene binding activity of ETR1[1–128]-GST at ~30% the activity observed with $CuSO_4$. Silver nitrate also supported similar levels of ethylene binding activity to ERS1[1–128]-GST but failed to support ethylene binding activity to the binding domains of the other three receptor isoforms (Fig. 4, supplemental Table S1). Similar results were obtained in four other experiments. Thus, silver ions support ethylene binding to ETR1 and ERS1 but not ETR2, ERS2, and EIN4.

ETR1 Promoter-driven Expression of Receptors and the Rescue of the Silver Phenotype—To further delineate the roles of the various receptor isoforms in the effects of silver, we transformed *etr1-6;etr2-3;ein4-4* triple mutants with cDNA con-

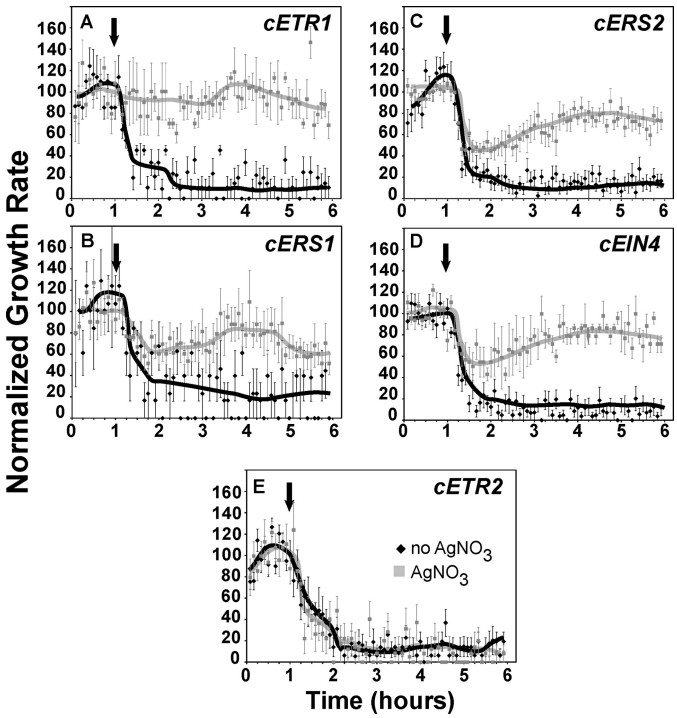

FIGURE 5. **The effect of silver nitrate on ethylene growth response kinetics of triple *etr1-6;etr2-3;ein4-4* mutants transformed with cDNA for *ETR1*, *ERS2*, *ERS2 EIN4*, or *ETR2*.** All constructs were under the promoter control of *ETR1*. Each panel (*A–E*) shows data for one seed line as designated. The transformed seedlings were grown in air for 1 h followed by application of 1 μl/liter ethylene (*arrow*) for 5 h. The hypocotyl response kinetics of seedlings grown on 100 μM silver nitrate are compared with seedlings grown in the absence of added silver nitrate. Data represent the mean ± S.E. from at least 5 seedlings total from at least four separate experiments. *Lines* were drawn by hand.

structs for each of the receptor isoforms from *Arabidopsis* and examined the ethylene growth responses in the absence and presence of 100 μM silver nitrate (Fig. 5). This triple mutant was chosen because silver nitrate had no obvious effect on ethylene responses in this mutant (Figs. 2 and 3*I*). In particular, the growth inhibition kinetics were nearly identical whether silver nitrate was present or not (Fig. 3*I*). To minimize effects from differential expression patterns, the cDNAs for all five receptor genes were placed under the control of the *ETR1* promoter. We have previously shown that all five transgenes are expressed and produce functional proteins in this mutant background (20).

Time-lapse imaging of these transformants showed that the *cETR1* transgene completely rescued the silver phenotype so that seedlings had no growth inhibition response when ethylene was applied in the presence of silver nitrate (Fig. 5*A*). Transformation with the *cERS1*, *cERS2*, or *cEIN4* transgenes resulted in seedlings that had a partial response to ethylene in the presence of silver nitrate that was characterized by a partial first phase and delayed or incomplete growth recovery to pretreatment rates in the continued presence of ethylene (Fig. 5, *B–D*). Particularly interesting is that transformation of the *etr1-6; etr2-3;ein4-4* triple mutant with the *cETR2* transgene failed to rescue the silver phenotype so that there was no obvious difference in the ethylene response kinetics in the presence or absence of silver nitrate (Fig. 5*E*). Similar results were observed with two other *cETR2* transformant lines (data not shown). This failure of *cETR2* to rescue the silver phenotype is not due to a poorly expressed or a non-functional gene product since *cETR2* is expressed at higher levels than either *cETR1* or *cEIN4* and it rescues other phenotypes including diminished growth in air (20). These results suggest that the importance of each receptor isoform in mediating responses to silver does not correlate with the ability of silver to incorporate into that isoform and support ethylene binding.

Ethylene Binding Affinity to ETR1 with Copper versus Silver Ions—As in the current study, we have previously noted that ETR1 receptors incubated with silver nitrate have ~30% the ethylene binding activity of ETR1 receptors incubated with $CuSO_4$ (32, 43). We further investigated this difference in the levels of ethylene binding with silver ions *versus* copper ions to gain a better idea of how silver ions affect ETR1. We examined the dissociation time-course of $^{14}C_2H_4$ from ETR1[1–128]-GST labeled with 0.1 μl/liter $^{14}C_2H_4$ in the presence of $CuSO_4$ or silver nitrate. Prior studies have shown that ethylene dissociation from intact yeast expressing either the full-length ETR1 or ETR1[1–128]-GST containing copper is slow with a half-time of ~12.5 h (15, 41). We found that membranes isolated from yeast expressing ETR1[1–128]-GST and incubated with $CuSO_4$ released ethylene with a half-time of ~12 h compared with an approximate 10 h half-time for release of ethylene for ETR1[1–128]-GST incubated with silver nitrate (Fig. 6*A*).

To further investigate the effects of silver ions on ETR1, we determined the K_d for ethylene. We compared the effects of $CuSO_4$ and silver nitrate on ethylene binding levels to ETR1[1–128]-GST treated with 1 μl/liter of $^{14}C_2H_4$ in the presence of increasing concentrations of $^{12}C_2H_4$ (Fig. 6*B*). Only minor differences were observed in the binding curves with either metal. Similar results were obtained in two additional assays (data not shown). Scatchard analysis of all three experiments using the methods of Sisler (51) yielded a K_d value of 1.24 ± 0.26 μl/liter of ethylene with $CuSO_4$ and 0.98 ± 0.19 μl/liter of ethylene with silver nitrate; these values did not differ significantly (p = 0.43). Thus, the reduced level of ethylene binding to ETR1 in the presence of silver nitrate is not due to reduced affinity of ethylene to the receptor in the presence of silver ions.

DISCUSSION

Silver nitrate is known to block ethylene responses in plants (34) yet support ethylene binding to the ETR1 receptor (32, 43). This has led to a model where the larger silver ion occupies the binding site and interacts with ethylene but prevents stimulus-response coupling through the receptors (19, 32, 43, 44).

Whereas silver ions have been shown to alter other processes such as auxin transport (59), the results presented here show that the ethylene receptors mediate the effects of silver on ethylene responses. Unexpectedly we found that ETR1 is sufficient and has the predominant role in blocking ethylene responses in dark-grown *Arabidopsis* seedlings. Loss-of-function *etr1* mutants had long-term reductions in responses to silver that were not seen with single loss-of-function mutants for the other receptor isoforms. These results correlate with the observation that ETR1 has a larger role than the other isoforms in ethylene signaling that leads to control of seedling growth (3, 45). Even though ETR1 has the major role in controlling silver

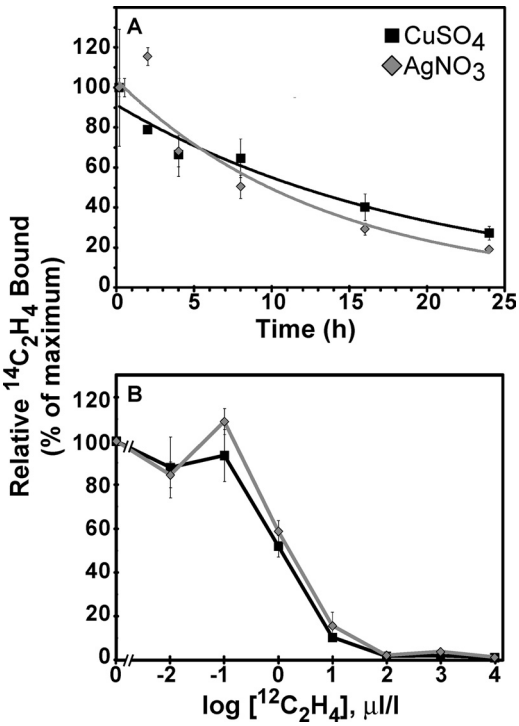

FIGURE 6. **Comparison of ethylene binding to ETR1[1–128]-GST with copper sulfate *versus* silver nitrate.** Ethylene binding was determined for membranes isolated from yeast expressing ETR1[1–128]-GST. Membranes were pre-incubated with either 300 μM copper sulfate or silver nitrate for 30 min prior to determining saturable ethylene binding. In both panels, the mean normalized level of $^{14}C_2H_4$ bound ± S.D. is shown. *A*, time course of $^{14}C_2H_4$ dissociation from ETR1[1–128]-GST was determined after binding was carried out with 0.1 μl/liter $^{14}C_2H_4$. Samples were aired for the indicated times in a chamber with a continuous flow of humidified air and analyzed for $^{14}C_2H_4$ remaining. Data for each incubation condition were normalized to the level of ethylene binding after airing for 10 min. *B*, ethylene binding levels with 1 μl/liter $^{14}C_2H_4$ in the presence of increasing amounts of $^{12}C_2H_4$ at the indicated concentrations were determined. Data were normalized in each condition to the levels of binding in the absence of added $^{12}C_2H_4$.

responses, our results indicate that the other isoforms also contribute to this trait. A slightly reduced response to silver was observed in *ers1-3*, *etr2-3*, and *ein4-4* loss-of-function mutants that was characterized by an attenuated first phase growth inhibition response when ethylene was added. Additionally, the *etr1* single loss-of-function mutants still had a partial response to silver while the *etr1-6;etr2-3;ein4-4* triple mutants had no response to silver indicating that other isoforms contribute to the silver phenotype.

Further support for the importance of ETR1 is our observation that transformation of the *etr1-6;etr2-3;ein4-4* triple mutant with a transgene for *ETR1* rescued the silver phenotype while transformation with cDNA for *ERS1*, *ERS2*, or *EIN4* only partially rescued the silver phenotype and *cETR2* failed to rescue the silver phenotype. It is unclear why this transgene was ineffective at rescuing this trait since it has previously been shown to express a functional protein that rescues other traits (20). These results show that there are differences in the receptors that are important for mediating responses to silver. One explanation for this could simply be that the isoforms are expressed at different levels or with different expression patterns in dark-grown *Arabidopsis* seedlings. However, this is not likely to be the entire explanation since *ERS1* is expressed at nearly the same levels as *ETR1* in dark-grown *Arabidopsis* seedlings (16) yet loss of ERS1 had a much smaller effect on responses to silver nitrate than loss of ETR1. Also, the cDNA transformants used in this study were under the promoter control of *ETR1* to limit differences due to variations in expression patterns. All of these transgenes were expressed at higher levels than *cETR1* (20) yet were less effective at rescuing the silver phenotype. Thus, there are functional differences between the receptor isoforms that impact responses to silver. We showed that silver nitrate only supports ethylene binding to subfamily I receptors indicating there are biochemical differences between the different isoforms that may be important in mediating the effects of silver on ethylene perception.

The fact that transgenes for *ETR1, ERS1*, *ERS2*, and *EIN4* can rescue or partially rescue the silver response in *etr1-6;etr2-3;ein4-4* triple mutants while silver only supports ethylene binding to ETR1 and ERS1 suggests that alternative mechanisms for the effects of silver need to be considered. One possibility is that silver binds to ERS2 and EIN4 but blocks ethylene binding to these isoforms. This seems unlikely since silver binds olefins, however, our data do not rule out this possibility. Another possibility is that silver nitrate affects the receptors outside of the binding domain to alter signaling. For instance, there is accumulating evidence that the ethylene receptor dimers function as higher order receptor clusters where the signaling state of one receptor dimer influences the signaling state of neighboring receptor dimers through direct physical interactions (9, 12, 16, 21, 44, 47, 54, 60–63). We have previously proposed that these interactions may underlie the high ethylene sensitivity observed for the first phase of growth inhibition (47). It is thus possible that silver ions affect receptor clustering to subtly enhance output of the receptors to reduce perception of ethylene. This would explain why, in the presence of silver nitrate, ethylene causes a partial growth inhibition response resulting in an attenuated first phase response in some of the receptor loss-of-function mutant combinations and transformants. An argument against this having a major role in this trait is that the *ers1-3;etr2-3;ein4-4;ers2-3* quadruple mutants that only contain the ETR1 ethylene receptor isoform still respond normally to the addition of silver nitrate. However, it is possible that it is the clustering of the ETR1 receptors that controls responses to silver ions with the other isoforms differentially modulating this clustering. Thus, silver ions may be having a second effect on the ethylene receptors leading to altered receptor clustering or signal output.

In this study we also examined the effects of silver nitrate on the ETR1 ethylene receptor. Similar to our previous findings, we found that silver nitrate only supported ~30% of the ethylene binding activity of ETR1 in the presence of $CuSO_4$ (32, 43). This reduced binding with silver nitrate is not due to suboptimal levels of silver since higher levels of silver nitrate do not increase levels of ethylene binding to ETR1 (43). Additionally, the lower binding of ethylene to ETR1 in the presence of silver nitrate is not due to a lower affinity to ethylene since similar K_d values for ethylene binding to ETR1[1–128]-GST were obtained with either metal. These observations are consistent with the suggestion that silver ions have characteristics of a non-competitive inhibitor of ethylene action (34) that would be

expected to have no effect on the affinity of the receptors for ethylene. Experimental and computational studies on Group 11 metal-olefin complexes predict that silver-olefin bonds have ~72% the bond dissociation energy of copper-olefin bonds (64–69). We therefore predicted that ethylene might have a faster half-time of release from ETR1 in the presence of silver nitrate compared with $CuSO_4$. Consistent with this prediction, the half-time of ethylene release from ETR1[1–128]-GST in the presence of silver nitrate was ~83% the half-time of release in the presence of $CuSO_4$. However, this reduction in the half-time of release is not enough to account for the much lower ethylene binding activity observed with silver nitrate. In addition to slow release kinetics, plants also have a rapid release of ethylene with a half-time of ~30 min (70, 71) that might be caused by receptor proteolysis (72). Our results show that the exogenously expressed receptors do not have this rapid ethylene release in agreement with prior results on exogenously expressed ethylene receptors (15, 41). Our first time point at 10 min showed that the receptors with silver already had reduced ethylene binding indicating that it is likely the receptors are binding less ethylene rather than releasing ethylene faster.

Thus, the lower ethylene binding activity observed in ETR1 treated with silver nitrate is not due to lower affinity to or faster release from ETR1 receptors containing silver. Therefore, we predict that the number of active ethylene binding sites is lower in ETR1 treated with silver nitrate than with $CuSO_4$. We previously observed that there was one copper per receptor dimer (32) that has led to a model where there is one copper ion per receptor dimer. However, we also noted in this prior study that not all the receptors were active and capable of binding ethylene (32). An alternative model is that each ETR1 receptor contains more than one copper per active receptor dimer with each copper capable of binding ethylene. In this model, we predict that each ETR1 receptor dimer only contains one silver and this leads to the lower levels of ethylene-binding activity in ETR1 receptors exposed to silver nitrate. Alternatively, it is possible that fewer ETR1 proteins contain a metal cofactor in the presence of silver nitrate *versus* $CuSO_4$. In either model, there are fewer ethylene binding sites generated in the presence of silver that would lead to a reduction in overall binding of ethylene without significantly affecting ethylene binding affinity. More refined analyses will be required to test these models.

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