

Subcellular Localization and In Vivo Interactions of the *Arabidopsis thaliana* Ethylene Receptor Family Members

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ABSTRACT The gaseous phytohormone ethylene regulates many developmental processes and responses to environmental conditions in higher plants. In *Arabidopsis thaliana*, ethylene perception and initiation of signaling are mediated by a family of five receptors which are related to prokaryotic two-component sensor histidine kinases. The transient expression of fluorescence-tagged receptors in tobacco (*Nicotiana benthamiana*) epidermal leaf cells demonstrated that all ethylene receptors are targeted to the ER endomembrane network and do not localize to the plasmalemma. In support of in planta overlay studies, the ethylene receptors form homomeric and heteromeric protein complexes at the ER in living plant cells, as shown by membrane recruitment assays. A comparable in vivo interaction pattern was found in the yeast mating-based split-ubiquitin system. The overlapping but distinct expression pattern of the ethylene receptor genes suggests a differential composition of the ethylene receptor complexes in different plant tissues. Our findings may have crucial functional implications on the ethylene receptor-mediated efficiency of hormone perception, induction of signaling, signal attenuation and output.

INTRODUCTION

In recent years, the molecular action of phytohormones like ethylene has been intensively studied in plants. The gaseous and diffusible molecule ethylene affects many aspects of the plant life, including seed germination, seedling development, abscission, organ senescence, fruit ripening and stomata opening (Abeles et al., 1992; Guo and Ecker, 2004; Desikan et al., 2005, 2006). Moreover, reactions of plants to biotic and abiotic stresses involve ethylene. Recently, several studies point out a complex interplay between signaling by ethylene and other plant hormones such as auxin (Li et al., 2004; Stepanova et al., 2005), gibberellins (Vriezen et al., 2004), brassinosteroids (De Grauwe et al., 2005) and cytokinin (Smets et al., 2005; Cho and Yoo, 2007), which also depends on the environmental condition and the plant's developmental stage.

Insights into the ethylene-response pathway have mainly arisen from molecular studies in the model plant *Arabidopsis thaliana* (*At*) using genetic screens based on an altered 'triple response' (summarized in Chen et al., 2005; Etheridge et al., 2006): in *Arabidopsis* ethylene is perceived by a family of five receptors (ETR1, ERS1, ETR2, ERS2, EIN4), which are related to prokaryotic, fungal and plant sensor histidine kinases (Hwang et al., 2002; Grefen and Harter, 2004). Based on phylogenetic analysis and the presence of conserved sequences in the histi-

dine kinase domain, the receptors are divided into two subfamilies (Hall and Bleecker, 2003). Subfamily I consists of ETR1 and ERS1 which have histidine kinase activity. Subfamily II is formed by ETR2, ERS2 and EIN4. The amino acid sequences of the subfamily II members lack residues considered to be essential for histidine kinase activity. In the absence of ethylene, the receptors maintain the activity of the MAPKKK CTR1, which interacts with the ethylene receptor complex (Gao et al., 2003) and represses downstream ethylene signaling in air. In the presence of ethylene, the receptors become inactivated, which leads to a proposed conformational change in CTR1, causing the suppression of its kinase activity and the de-repression of the ethylene response pathway. The signaling events downstream of CTR1 are mediated by a potential and debated MAP kinase cascade—the Nramp metal ion transporter-like protein EIN2 followed by members of the EIN3/EIL family of transcription factors. The EIN3/EIL transcription

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factors differentially regulate the transcription of primary ethylene response genes which induce a transcriptional cascade responsible for the realization of the plant's reactions to exogenously applied hormone (Chen et al., 2005).

However, there also appear to exist CTR1-independent, two-component system (TCS) element-dependent signaling pathways which are regulated by the histidine kinase activity of the subfamily I members ETR1 and ERS1. The histidine kinase activity of ETR1 was demonstrated to be important in the rate of growth recovery after ethylene removal (Binder et al., 2004a) and in the endogenous ethylene-regulated promotion of *Arabidopsis* growth (Cho and Yoo, 2007). Furthermore, it was shown that ETR1-dependent phosphorylation of the B-type response regulator ARR2 activates ethylene response transcription (Hass et al., 2004), and that ETR1 is able to induce two component signaling events in vivo (Cho and Yoo, 2007). Although the CTR1-independent and TCS-related pathway provides only a minor contribution to the realization of the exogenous ethylene-controlled hypocotyl growth response in etiolated seedlings (Hass et al., 2004; Mason et al., 2005; Cho and Yoo, 2007), it appears to play a crucial role in the ethylene-regulated stomatal closure (Desikan et al., 2006). The functional relevance of the histidine kinase activity and an alternative pathway was recently supported by the observation that the subfamily I receptors ETR1 and ERS1 play a predominant role in mediating ethylene responses (Qu et al., 2007).

Genetic studies in *Arabidopsis* and other plant species have shown that gain-of-function mutations in any of the five receptors confer ethylene insensitivity (Bleecker and Schaller, 1996; Gamble et al., 2002). Furthermore, physiological investigations demonstrated that ethylene effects in plants are observed at extremely low concentrations and over a concentration range of seven orders of magnitude (Chen and Bleecker, 1995; Binder et al., 2004a, 2004b). This suggests that the ethylene perception system has evolved signal-amplification mechanisms that enable the recognition of very subtle changes in the number of hormone-occupied receptors and the transformation of the system into a state that allows the efficient induction of the response pathway. In analogy to the bacterial chemotaxis histidine kinase receptors (Gestwicki and Kiessling, 2002; Thomason et al., 2002), one possible mechanism is that a few ethylene-occupied, active receptors recruit by physical interaction with neighboring receptors to higher-order protein clusters. Through direct contact, the active receptors can influence the signaling state of the neighbors within the cluster. This could result in the coordinated transformation of multiple receptors into active states by a few ethylene-binding events. However, such a mechanism implicates that all members of the ethylene receptor family are targeted to the same intracellular compartment and physically interact at this subcellular site. A candidate compartment is the ER network, to which at least ETR1, ETR2 and the *Cucumis melo* ortholog of the *Arabidopsis* ERS1, *CmERS1*, and CTR1 are targeted or recruited to (Chen et al., 2002, 2007; Gao et al., 2003; Ma et al., 2006).

Here, we provide for the first time cell biological evidence that all *Arabidopsis* ethylene receptors are located in the ER endomembrane system in living plant cells. Furthermore, by using a novel in planta membrane recruitment assay (MeRA) and the mating based split-ubiquitin system (mbSUS; Johnsson and Varshavsky, 1994; Obrdlik et al., 2004), we show that the receptors form homo- and heteromeric complexes in any combination in vivo. The analysis of the steady-state transcript level by semi-quantitative RT-PCR and *ETR1* and *ERS1* gene activity using *promoter::uidA* reporter lines indicates an overlapping but distinct expression of the receptors in *Arabidopsis*. The latter suggests a tissue-specific configuration of ethylene receptor complexes. We discuss the functional implications of our observations in the light of ethylene perception, receptor function and initiation of signaling.

RESULTS

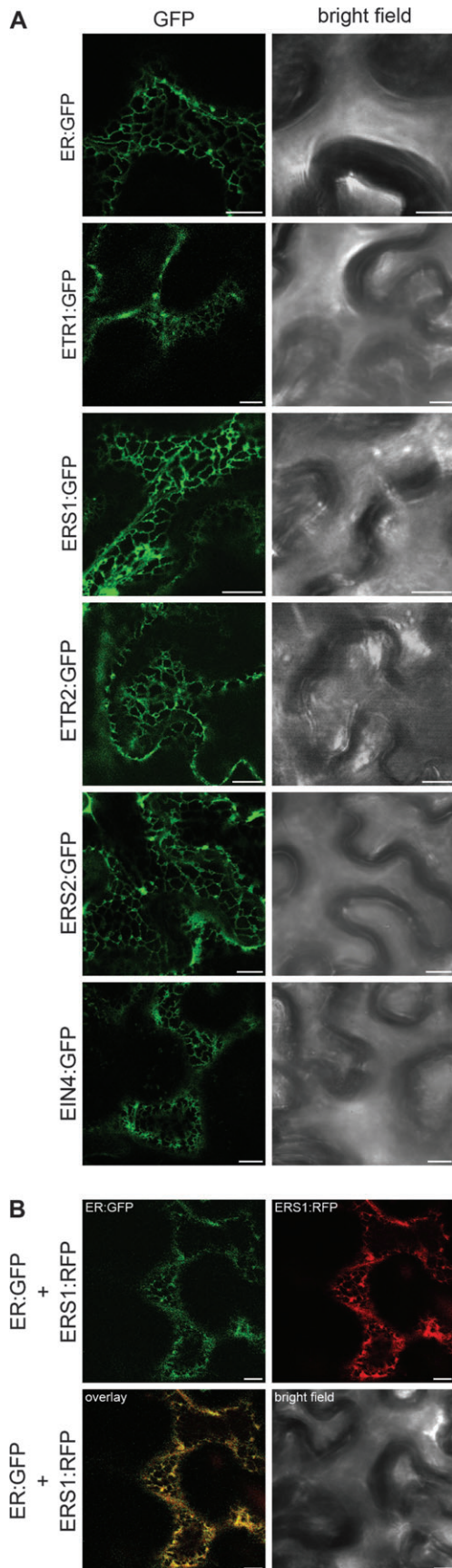
The *Arabidopsis* Ethylene Receptors are Localized in the Endoplasmic Reticulum Membrane (ER) of Plant Cells

In order to investigate the subcellular localization of the *Arabidopsis* ethylene receptors, we fused the fluorescent proteins GFP or RFP to the C-terminus of all five *Arabidopsis* ethylene receptors and expressed the fusion proteins under the control of the constitutive 35S promoter. The repeated transient transfection of these constructs into *Arabidopsis* protoplast yielded no detectable fluorescence signals, probably due to strong transgene silencing. To overcome this problem, we chose to examine the localization of the receptors in *Agrobacterium*-infiltrated *Nicotiana benthamiana* leaf cells. The co-infiltration of the silencing inhibitor gene *p19* from the tomato bushy stunt virus (TBSV) enables the efficient expression of many plant proteins in this system (Voinnet et al., 2003). After transformation, the abaxial epidermis of the tobacco leaves was subjected to confocal laser scanning microscopy (CLSM). As shown in Figure 1A, a reticular pattern was observed for all five ethylene receptors. The identity of the stained endomembrane system as ER was verified by the identical localization pattern of an ER marker protein (Figure 1A). Please note that we focused at the cytoplasmic surfaces of the transformed cells in these images.

The ER localization of ERS1 was directly tested by co-expression with the ER marker protein. A clear co-localization of ERS1 and the ER marker was observed, as indicated by the perfect overlay of the RFP and GFP fluorescent signals (Figure 1B). Furthermore, a comprehensive CSLM overlay study revealed a strict co-localization of all five ethylene receptors in the ER (Supplementary Figure 1).

The Ethylene Receptors are not Detectable in the Plasmalemma of Plant Cells

We next examined whether the ethylene receptors are exclusively localized in the ER or whether a detectable fraction is also found in the plasmalemma. Therefore, the RFP-tagged



ethylene receptors were co-expressed in tobacco leaves with the GFP-tagged brassinosteroid receptor BRI1, which has been shown to be a plasmalemma protein (Friedrichsen et al., 2000). In the case of a plasmalemma-bound protein, a continuous fluorescence signal is expected in cross-section images of transformed cells. In contrast, a discontinuous fluorescence signal would be typical for the reticular structure of the ER. Additionally, a fluorescence signal should also be detected around the nucleus, as the nuclear membrane forms a continuum with the ER. Cross-section images of tobacco epidermal cells that co-expressed the ethylene receptor RFP fusions with BRI1-GFP showed always a discontinuous RFP signal, whereas the GFP signal was continuous (Figure 2). An RFP signal was also detected around the nucleus, as exemplarily shown for ERS1, ERS2, and EIN4 (Figure 2). When the RFP and GFP signals were overlaid, the separate localization of the ethylene receptor-RFP fusions and BRI1-GFP in the ER and the plasmalemma was confirmed (Figure 2). These data support the absence of detectable amounts of ethylene receptor fusion proteins in the plasmalemma and their localization in the ER endomembrane system.

The *Arabidopsis* Ethylene Receptors Show Overlapping but Distinct Expression Pattern

To investigate whether the five ethylene receptors may co-localize and, thus, co-function in planta, we analysed their steady-state transcript levels in different *Arabidopsis* tissues and at different developmental stages by semi-quantitative RT-PCR. The PCR was carried out with ethylene receptor gene-specific primers at a different number of cycles to enable a quantitative comparison of the PCR product with *ACTIN2*, as described previously (Horák et al., 2003). The amount of *ACTIN2* product proved that all samples contained similar amounts of cDNA, with the exception of the siliques, where the weaker intensity of the band reflects a lower expression of the gene in this tissue according to public microarray data (Figure 3A; eFP browser, <http://bbc.botany.utoronto.ca/efp/development/>). The *ERS1* gene had the highest transcript level and the most ubiquitous expression pattern among all of the studied ethylene receptors. The other four *Arabidopsis* ethylene receptors can be divided into two different groups: *ETR1* and *EIN4* are less expressed in etiolated seedlings and siliques. In contrast, *ETR2* and *ERS2* showed increased transcript levels in etiolated seedlings and decreased amounts in light-grown

Figure 1. The *Arabidopsis* Ethylene Receptors Localize to the ER Network in Transiently Transformed Tobacco Leaf Cells.

(A) Confocal images of abaxial epidermal leaf cells (surface focus) expressing the indicated GFP fusion proteins. ER:GFP represents an ER marker fusion protein. The right column shows the bright field images of the transformed cells.

(B) Confocal images of the co-expression of ERS1:RFP and ER:GFP. The yellow overlay color of the GFP and RFP fluorescence demonstrates the co-localization of both proteins in the ER.

The bars represent 10 μ m.

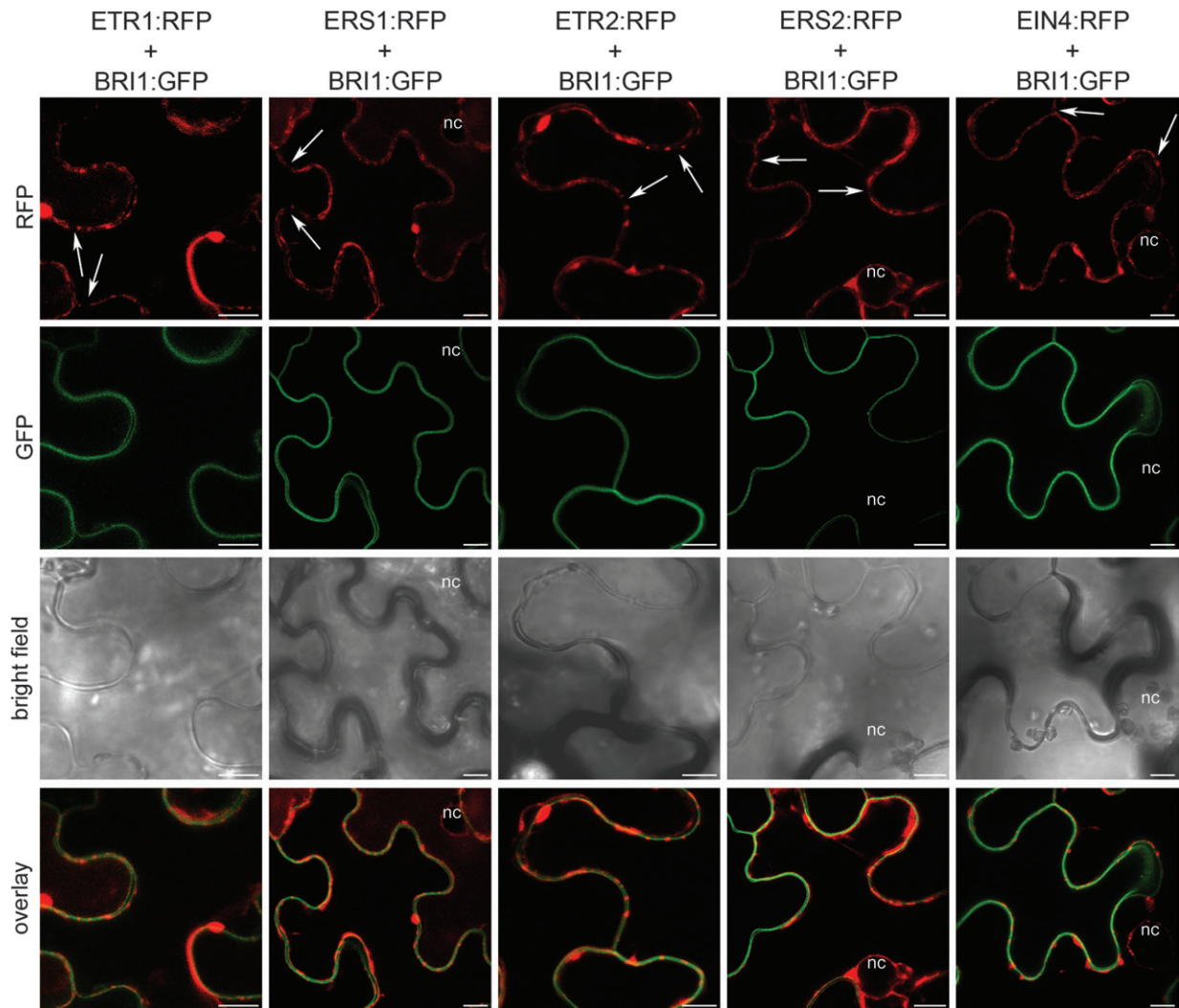
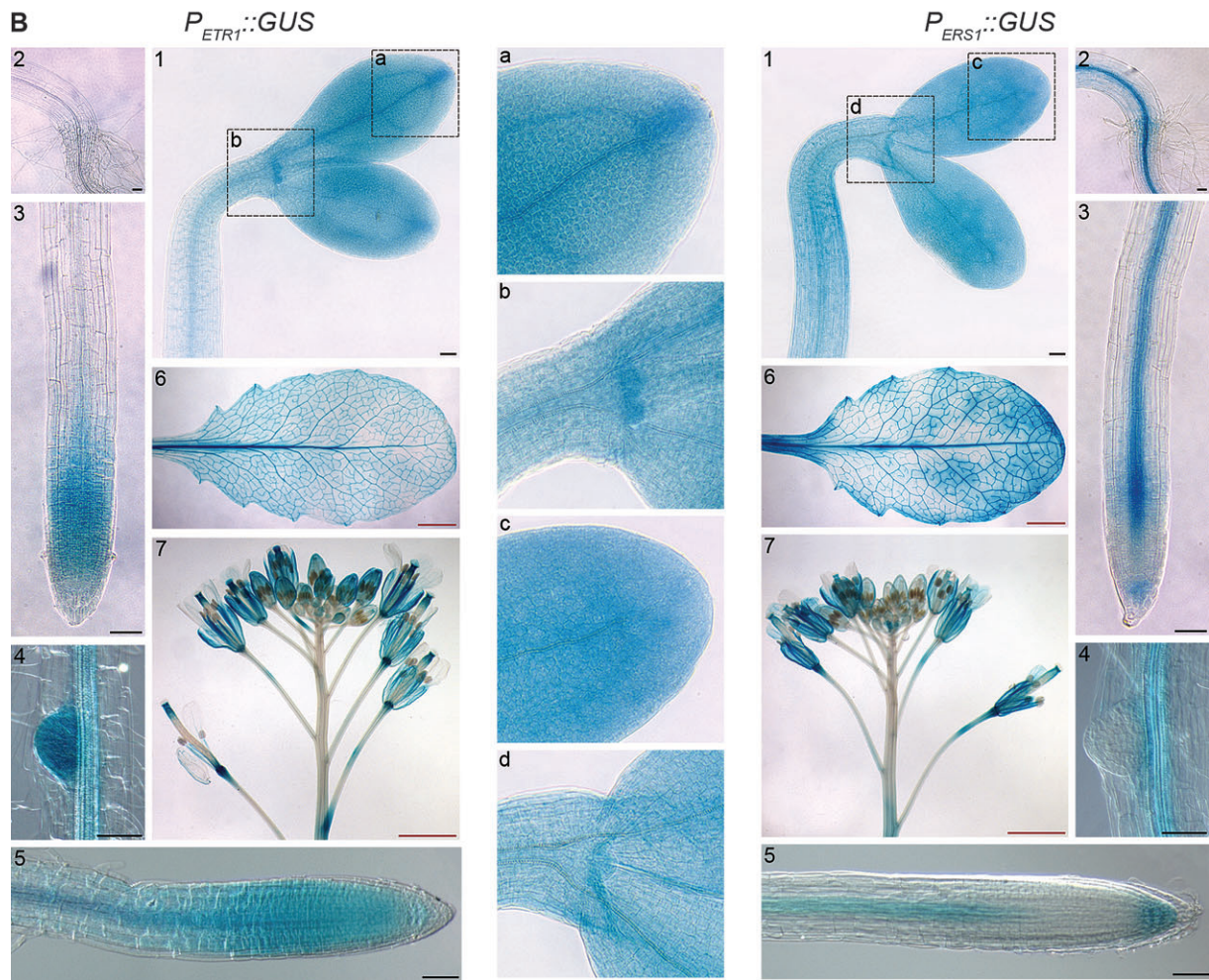
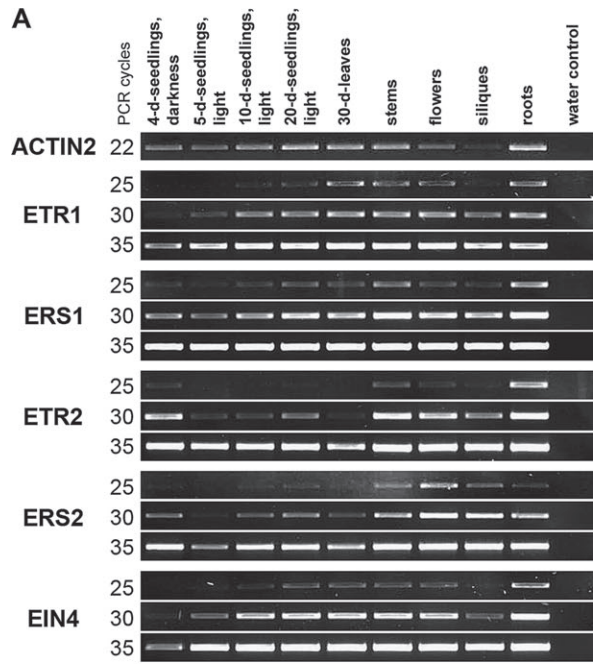


Figure 2. The *Arabidopsis* Ethylene Receptors are not Detectable in the Plasmalemma of Transiently Transformed Tobacco Leaf Cells. Confocal fluorescence, bright field and overlay images of abaxial epidermal leaf cells (cross-section focus) expressing the indicated GFP and RFP fusion proteins. The emission channels for the RFP and GFP fluorescence and the overlay are indicated at the left. The RFP signal of the ethylene receptors shows a discontinuous fluorescence pattern (white arrows) while the GFP signal of the BRI1 plasmalemma marker is continuous. The RFP fluorescence around the nucleus (nc) is detectable for ERS1, ERS2 and EIN4. For overlay images, the intensity of the RFP signal was slightly enhanced. The bars represent 10 μ m.

seedlings and leaves. However, transcripts of all ethylene receptors were detected in all tested tissues and developmental stages.

To extend our analysis towards the activity of the ethylene receptor genes, we translationally fused the promoters of the functionally most important subfamily I ethylene receptors *ETR1* and *ERS1* (P_{ETR1} , P_{ERS1}) to the *uidA* reporter gene and introduced the constructs into *Arabidopsis*. $P_{ETR1}::GUS$ activity was detected in the cotyledons and hypocotyl vascular tissue of 3 d old dark-grown seedlings, rosette leaves, flowers of 30 d old plants and the meristematic and proximal elongation zone of the root of etiolated and 7 d old light-grown seedlings (Figure 3B; $P_{ETR1}::GUS$, images 1, 3, 5–7). Moreover, *ETR1* promoter activity was observed in the lateral root primordia (Fig-

ure 3B; $P_{ETR1}::GUS$, image 4), and enhanced in the cotyledon tip and the shoot meristem (Figure 3B, enlarged images a, b). No GUS activity was observed in the hypocotyl/root junction of etiolated seedlings (Figure 3B; $P_{ETR1}::GUS$, image 2). The expression pattern of $P_{ERS1}::GUS$ was similar to that of $P_{ETR1}::GUS$ (Figure 3B, $P_{ERS1}::GUS$), with a few, but significant, differences: in contrast to *ETR1*, *ERS1* promoter activity was detected in the hypocotyl/root junction of dark-grown seedlings (Figure 3B; $P_{ERS1}::GUS$, image 2) but restricted to the stele and the root cap of etiolated and light-grown seedlings (Figure 3B; $P_{ERS1}::GUS$, images 3, 5) and was not found in the lateral root primordia (Figure 3B; $P_{ERS1}::GUS$, image 4). Compared to $P_{ETR1}::GUS$, a lower *ERS1* promoter activity was observed in the vegetative meristem and the cotyledon tip (Figure 3B; enlarged images c,



d). Common to both promoters is their activation in the abscission zones during flower senescence and silique development (Figure 3B; image 7). The observed expression pattern is in agreement with microarray data available in the public databases, such as the eFP browser (<http://bbc.botany.utoronto.ca/efp/development/>). In conclusion, the tested subfamily I ethylene receptors *ETR1* and *ERS1* show an overlapping but also a distinct expression pattern in the root.

The Ethylene Receptors Interact in Living Plant Cells

The co-expression and co-localization strongly suggest that the ethylene receptors might physically associate in the ER membrane network. However, although the homomeric interaction of yeast-expressed full-length *ETR1* was shown previously by a biochemical approach (Schaller et al., 1995) and the corresponding N- and C-terminal split-YFP fusion proteins were properly expressed in tobacco cells (data not shown), all of our BiFC assays (Walter et al., 2004) failed to prove the *in vivo* interaction of the ethylene receptors. We therefore developed a novel approach to study protein–protein interactions in planta—the membrane recruitment assay (MeRA), which is a useful alternative to BiFC (and FRET). MeRA is based on a RFP-fluorescent anchor protein which is localized in a membrane compartment such as the ER and a cytoplasmic GFP- (or YFP-) fluorescent ‘prey’ protein which shows a diffusive intracellular fluorescent pattern (Figure 4A, hypothesis). When both proteins interact, the membrane-bound anchor protein recruits the cytoplasmic fusion protein to the well defined and easy-to-identify membrane compartment (Figure 4A, hypothesis).

We therefore used the MeRA to study the interaction of the ethylene receptors in living plant cells (Figure 4A and 4B). The CLSM images are exemplarily shown for the interaction of the ER-bound full-length *ERS1*:RFP (anchor protein) and the transmembrane domain-lacking (Δ TM) cytoplasmic versions of *ETR2*:GFP and several controls (Figure 4B). In the absence of the *ERS1*-RFP anchor protein, Δ TM-*ETR2*:GFP showed a diffused cytoplasmic fluorescence pattern (Figure 4B, left column). In contrast, when *ERS1*:RFP was present in the cell, Δ TM-*ETR2*:GFP was recruited to the ER (Figure 4B, fourth column). *ERS1*:RFP did not recruit GFP to the ER (Figure 4B, second column). Furthermore, when the ER–marker–RFP fusion was co-expressed with Δ TM-*ETR2*:GFP, a recruitment of the ethylene receptor to the ER was also not observed (Figure 4B, third column). These data indicate that the RFP-tag per se does not have unspecific recruitment activity and, thus, that the inter-

action of *ERS1* and Δ TM-*ETR2* is specific. A comprehensive MeRA interaction analysis revealed that the *Arabidopsis* ethylene receptors formed homo- and heteromeric complexes in all possible combinations in the ER of living plant cells (Figure 4C).

Furthermore, we tested whether the recruitment activity of *ERS1*:RFP depends on the presence of the GAF domain in Δ TM-*ERS1*:GFP. *ERS1*:RFP was not able to recruit a mutant version of Δ TM-*ERS1*:GFP to the ER which lacked the GAF domain (Δ TM/GAF-*ERS1*:GFP; Figure 4A, right column). Thus, the presence of the GAF domain is critical for the homomeric interaction of *ERS1* in living plant cells.

Homo- and Heterodimerization of the Ethylene Receptors in Yeast

To support the interaction pattern observed in our MeRA study, we performed a comprehensive interaction analysis using the mating-based split-ubiquitin system (mbSUS; Obrdlik et al., 2004; Grefen et al., 2007). Therefore, the full-length ethylene receptors were fused to the C-terminal ubiquitin (Cub-PLV) fragment and transformed in the haploid yeast strain THY.AP4 (MAT a). The membrane domain-lacking (Δ TM) ethylene receptor versions used in the MeRA approach were fused to N-terminal ubiquitin (Nub) fragment and transformed in the haploid yeast strain THY.AP5 (MAT α). Prior mating the correct expression of the ethylene receptor fusion proteins was verified in the haploid yeast strains by western blotting using PLV- and HA-specific antibodies (Figure 5A). After mating of the fusion protein-expressing yeast cells, the presence of the plasmids (Figure 5B, right) and the interaction of the ethylene receptors (Figure 5B left) were assayed by growth on the appropriate media. All yeast transformants grew on the plasmid-selective media, indicating that the ethylene receptor fusion proteins are not toxic (Figure 5B, left). Furthermore, any ethylene receptor combination tested induced the growth of yeast cells on interaction selective media (Figure 5B, right). In contrast, no growth was observed when the receptors were co-expressed with the empty vector (Figure 5B, right; NubG). The presence of the full-length ethylene receptors in the diploid yeast cells was verified by their interaction with the wild-type Nub (NubWt), which unspecifically associates with the Cub fragment (Obrdlik et al., 2004; Grefen et al., 2007). Thus, comparable to the data obtained by in-planta MeRA, the members of the *Arabidopsis* ethylene receptor family can homo- and heterodimerize in any combination in yeast.

Figure 3. The Ethylene Receptors Show an Overlapping but Distinct Expression Pattern in *Arabidopsis*

(A) Expression analysis of the ethylene receptors in different tissues and developmental stages by semi-quantitative RT-PCR. cDNA was derived from the indicated tissues and developmental stages and used as a template for PCR with the listed gene specific primers at different numbers of PCR cycles. PCR products were visualized by agarose gel electrophoresis and ethidium bromide staining.
(B) Histochemical staining of *P_{ETR1}::GUS* and *P_{ERS1}::GUS* activity in *Arabidopsis* tissues at different developmental stages. Three-day-old etiolated seedlings: 1, cotyledon and upper hypocotyl area; 2, hypocotyl / root junction; 3, root. Seven-day-old light-grown seedlings: 4, lateral root primordia; 5, root. Thirty-day-old plants: 6, rosette leaf; 7, inflorescence. a, b, c, and d are magnifications of the corresponding cotyledon/upper hypocotyl area. The red bars represent 3 mm and the black bars 100 μ m.

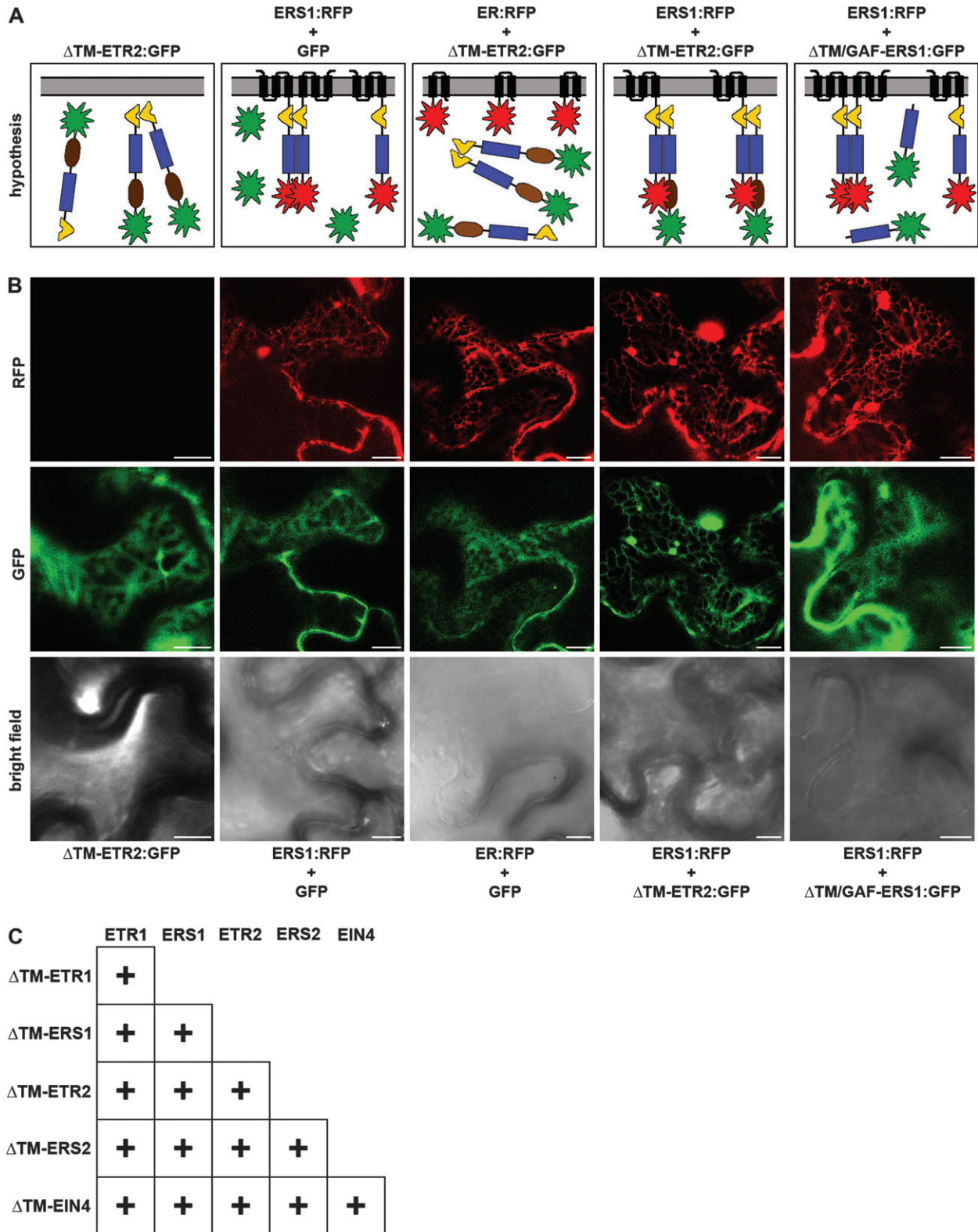


Figure 4. The Ethylene Receptors Interact at the ER Membrane in Transiently Transformed Tobacco Leaf Cells. **(A)** Principle of the membrane recruitment assay (MeRA) and scheme of the hypothetical ethylene receptor interaction pattern (hypothesis). Grey, ER membrane; black, transmembrane domain; yellow, GAF domain; blue, histidine kinase (-like) domain; brown, receiver domain; red, RFP-tag; green, GFP-tag.

DISCUSSION

The *Arabidopsis* Ethylene Receptors are ER-Localized Proteins

In our study, we present for the first time cell biological data which demonstrates that all *Arabidopsis* ethylene receptors localize to the ER in plant cells and confirm by in vivo experiments earlier biochemical findings for ETR1 and ETR2 (Chen et al., 2002, 2007). This intracellular distribution was observed independently of whether the fluorescence protein was fused to the N-terminus or the C-terminus of the receptors (J. Horák and K. Harter, unpublished results), indicating that the tag does not mislocalize the proteins. Furthermore, our control experiments diminish the possibility of the location of the receptors in other membrane compartments, including the plasmalemma. In conclusion, our results support earlier findings that the ER endomembrane network is the primary intracellular location of ethylene perception in plants.

Although we used the identical constructs for the transient transformation assays, the expression of fluorescence-tagged ethylene receptors failed in *Arabidopsis* cell culture protoplasts and was only detectable in *Agrobacterium*-infiltrated tobacco leaf cells in the presence of the silencing suppressor protein p19 (Voinnet et al., 2003). The *Arabidopsis* protoplasts are either capable to generally silence the transgenes or dra-

matically attenuate the ethylene-sensing machinery as a reaction to high ethylene levels which are generated in response to wounding during protoplast preparation (Yanagisawa et al., 2003).

Similarly, we were not able to achieve the overexpression of the ethylene receptors in transgenic *Arabidopsis* plants stably transformed with the identical GFP fusion constructs used for the transient assays. Although the fusion genes were under the control of the strong and constitutive 35S promoter, the transgenic plants managed to repress the expression of the fusion proteins to the transcript level of wild-type plants (Supplementary Figure 2A). Thus, the total RNA level of the ethylene receptors is tightly controlled in transgenic *Arabidopsis* plants by a post-transcriptional feedback mechanism. At this low expression level, we were not able to detect fluorescence in the transgenic plants. However, although with low penetrance, we regularly observed a premature leaf senescence phenotype in F1 transgenic plants which contained the ethylene receptor transgenes (Supplementary Figure 2B). Whilst more dramatic, a similar premature leaf senescence phenotype was recently described for *etr1/ers1* double loss-of-function mutants (*etr1-9/ers1-3*, *etr1-7/ers1-3*; Qu et al., 2007). This similarity suggests that the fluorescence protein-tagged ethylene receptors exert a dominant negative effect on the activity of the endogenous family members. Such interference can, however, only

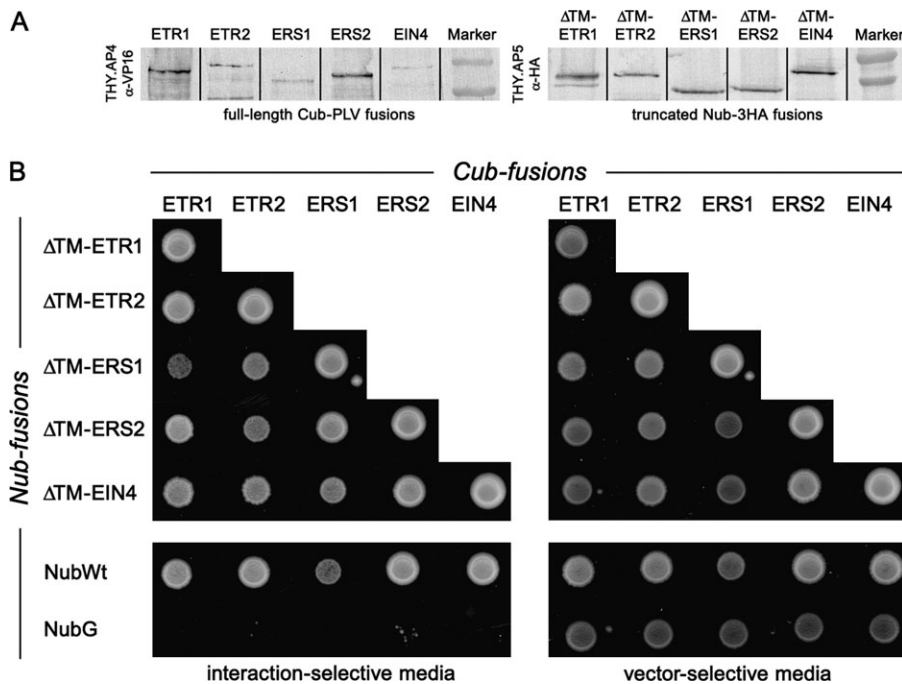


Figure 5. In-Vivo Interaction of the Ethylene Receptors in the Yeast Mating-Based Split-Ubiquitin System (mbSUS).

(A) Western blot analysis of total extracts derived from haploid yeast cells (THY.AP4 and THY.AP5) expressing the indicated ethylene receptor fusion proteins. Immunodetection of the fusion proteins were carried out with an antibody against the VP16-tag (α -VP16) and HA-tag (α -HA), respectively. The molecular mass of the markers are 150 and 100 kDa on the left and 100 and 75 kDa on the right. **(B)** Yeast mbSUS growth assays. Yeast THY.AP4 clones expressing the Cub-PLV-tagged full-length ethylene receptors were mated with THY.AP5 clones expressing the transmembrane-lacking (Δ TM) Nub-3HA-tagged receptor derivatives. The diploid cells were grown at 28°C either for 7 d on interaction- (left) or 3 d on vector-selective (right) media. NubWT was used as a positive, NubG as a negative control.

(B) Confocal images of abaxial epidermal leaf cells (surface focus) expressing the indicated GFP and RFP fusion proteins. The emission channels for the RFP and GFP fluorescence are indicated on the left. The lowest row shows the bright field images of the transformed cells. Δ TM, lack of the transmembrane domain; Δ TM/GAF, lack of the transmembrane and the GAF domain. The bars represent 10 μ m.

(C) MeRA-based matrix of the ethylene receptor interaction pattern.

occur when the dominant-negative and wild-type receptor variants function from the identical cellular compartment. According to the most recent model of ethylene receptor signaling (Qu et al., 2007), the fluorescent protein-tagged and dominant-negative-acting ethylene receptors might reduce the activity of the endogenous receptors to act on CTR1. This would lead to the inactivation of CTR1 and the activation of ethylene response pathway in air, which is reflected by the premature leaf senescence. It should be noted that the fluorescent tag of the ethylene receptors is attached to their C-terminus which is the interaction site for CTR1 (Clark et al., 1998; Gao et al., 2003). However, we cannot entirely exclude the possible mechanism that the 35S promoter-generated transcript of the ethylene receptor–GFP fusion itself competes with the corresponding mRNA of the endogenous gene which also would lead to a reduced number of functional wild-type receptor proteins in the ER.

The observation that all *Arabidopsis* receptors are targeted to the ER (shown here) and that CTR1—the immediate downstream signaling component of the receptors—is recruited by the receptors to the ER (Gao et al., 2003) strongly suggest that this endomembrane system is the general intracellular site for the initiation of ethylene signaling.

***Arabidopsis* Ethylene Receptor Interactions and the Role of the GAF Domain**

We demonstrated the interaction of all five *Arabidopsis* receptor family members in any combination by in-planta MeRA and yeast mbSUS, as hypothesized earlier (O'Malley et al., 2005). Surprisingly, our comprehensive in-planta BIFC and yeast mbSUS analysis failed to demonstrate the association of the full-length receptors, although the corresponding C-terminal split-YFP- and split-ubiquitin-tagged fusion proteins were properly expressed (data not shown). The most likely explanation for this discrepancy is that in the context of the full-length protein, the cytoplasmic domains of the receptors are less flexible or structurally arranged in a way that a close distance of the split-YFP- and split-ubiquitin-tagged monomers is not accomplished, which is necessary for the functional complementation of the YFP fluorophore or ubiquitin.

The results of our MeRA interaction analysis in tobacco cells expressing ERS1:RFP, Δ TM-ERS1:GFP and Δ TM/GAF-ERS1:GFP demonstrated that the interaction capability of at least the cytoplasmic extension of the ethylene receptor depends on the GAF domain in living plant cells. A comparable GAF domain-dependent interaction was recently reported for various soluble Δ TM fragments of *Arabidopsis* ETR1 in the yeast two-hybrid system (Xie et al., 2006). As shown for several other organisms, the GAF domain is capable of mediating reversible protein–protein interactions and activation of enzymatic activity (Aravind and Ponting, 1997; Ho et al., 2000; Martinez et al., 2002). Although the formation of covalent, non-reversible disulfide bounds in the N-terminal transmembrane domain was proposed to be relevant for ETR1 homodimerization

(Schaller et al., 1995), Xie and colleagues (2006) showed by a functional approach in transgenic *Arabidopsis* plants that this biochemical modification appears to be dispensable for ETR1 activity.

In conclusion, our results and the data of Xie et al. (2006) suggest that the GAF domain is the amino acid stretch which predominantly mediates non-covalent and reversible receptor association in vivo. However, we cannot exclude the possibility that the transmembrane domain also contributes to the interaction of the ethylene receptors. Further cell biological and functional studies are required to determine the function of the transmembrane and GAF domain for the dynamics of ethylene receptor association.

Interactions and Differential Expression of Ethylene Receptors: Functional Implications

As shown here, the five *Arabidopsis* ethylene receptors form heteromeric and homomeric protein complexes in vivo. With our cell biological techniques, we are not able to differentiate whether the observed interactions represent receptor homo- and heterodimers and/or homo- and heteromeric higher-order receptor complexes and their appearance in the different *Arabidopsis* tissues. In any case, the detected interaction capacity could provide the molecular basis for inter-receptor signaling which is responsible for the lack of a simple additive relationship between ligand binding and ethylene receptor outputs (O'Malley et al., 2005). Furthermore, it explains the high sensitivity and broad concentration range of ethylene responses and the observation that gain-of-function mutations in any ethylene receptor lead to the almost complete ethylene insensitivity in the otherwise wild-type background (Bleecker and Schaller, 1996; Gamble et al., 2002; Gehret et al., 2006). In analogy to the action of the chemotaxis receptors in bacteria (Gestwicki and Kiessling, 2002; Thomason et al., 2002), a mutant dominant-active ethylene receptor could be able to maintain the signaling state of the other family members, resulting in a constitutively high activity of CTR1. This would result in the continued repression of the ethylene response pathway, even in the presence of ethylene. Furthermore, especially at very low hormone concentrations, the activation of few receptor molecules could influence the activity state of the neighbors, enabling the efficient perception of the hormone over the reported seven orders of magnitude (Chen and Bleecker, 1995; Binder et al., 2004a, 2004b).

Our expression studies using semi-quantitative RT–PCR and transgenic *Arabidopsis* plants containing $P_{ETR1}::GUS$ and $P_{ERS1}::GUS$ show an overlapping but distinctive steady-state mRNA and gene activity pattern. For instance, whereas the activity of P_{ERS1} is restricted to the root tip and vascular tissue of 7 d old light-grown and 3 d old etiolated seedlings, P_{ETR1} activity is found in the lateral root primordia and the elongation zone.

The differential expression pattern in combination with the capability for homo- and heteromeric interaction and

ethylene-induced degradation (Chen et al., 2007) indicates that different plant tissues contain a distinct set of ethylene receptor complexes, whose composition may change during development and hormone treatment. Depending especially on the relative contribution of the functionally predominant class I receptors ETR1 and ERS1 (Qu et al., 2007), the complexes may possess differential in-plant affinity to ethylene, ETR1 (Clark et al., 1998; Cancel and Larsen, 2002), TCS signaling elements (Hass et al., 2004; Cho and Yoo, 2007) or putative membrane-intrinsic structural components such as RTE1 (Resnick et al., 2006). Therefore, a differential complex composition could result in differences in the signal-induced formation of higher-order receptor clusters, signaling efficiencies or signal attenuation. Moreover, the receptor complexes could also differ in other molecular features, such as membrane trafficking and internalization, feedback modification, turnover rate or recovery. Thus, one would predict a differential competence and responsiveness of plant tissues to ethylene, which depends on the intracellular level and composition of the receptor complexes.

Homo- and heteromeric ternary complex formation-dependent ligand binding and functional activity is well described for animal transmembrane receptor families such as the ErbB receptors (Carraway and Carraway, 2007). From the four members of the Erb family, only ErbB1 and ErbB4 are able to bind the high-affinity ligand and have a functional tyrosine kinase domain. ErbB2 cannot bind the ligand and ErbB3 has no functional kinase domain. However, every ErbB heterodimer is able to initiate signaling as long as it is composed of a signal sensing and phosphorylation-active monomer. The heterodimerization of ErbB receptors also permits a greater diversity of downstream responses regulating organism development and promoting oncogenesis (Carraway and Carraway, 2007).

Our results show that all five *Arabidopsis* ethylene receptors are targeted to the ER network in plant cells, form homo- and heteromeric protein complexes in vivo, and show an overlapping but distinct expression pattern, adding an additional level of complexity to ethylene perception and signal transduction. To define the specific biochemical features and functional attributes of distinct ethylene receptor complexes in a given tissue is a major scientific challenge for the future.

Moreover, by the means of molecular and cell biological approaches using fluorescence-tagged ethylene receptors, it will now be possible to investigate signal-dependent receptor complex formation, movement, internalization, and recovery in living plant cells. This will provide novel insights into the intracellular dynamics and function of this important plant receptor family.

METHODS

Plant Materials and Growth Conditions

Nicotiana benthamiana plants were cultivated in the greenhouse (temperature: day 25°C, night 19°C, humidity 60%,

lighting period 7:00–21:00). *Arabidopsis thaliana* Columbia ecotype (Col-0) was used as wild-type and cultivated in the greenhouse (temperature: day 21°C, night 18°C, humidity 45%, lighting period 7:00–21:00). *Arabidopsis* seedlings were cultivated at sterile conditions for histochemical staining and selection of transformants. Vapor sterilization of seeds was performed by placing seeds in opened Eppendorf tubes in a desiccator jar. A 250-ml beaker containing 100 ml of 12% sodium hypochlorite was placed inside and 10 ml concentrated HCl was added. The desiccator jar was closed and the seeds were sterilized through the nascent chlorine for 2 h. After sterilization, the Eppendorf tubes were placed under the fume hood for another 2 h to allow evaporation of remaining chlorine. Seedlings were cultivated in a growth chamber at 21°C under long-day conditions on 0.5 Murashige-Skoog medium, 1% (w/v) sucrose and 0.8% (w/v) phytoagar. Transformed seedlings were selected on media supplemented with 25 µg ml⁻¹ hygromycin B for 14 d and resistant plants were propagated on soil.

Cloning Strategies

All clones used in this article were constructed using Gateway™ technology (Invitrogen). The Entry clones were either obtained via BP-reaction in pDONR201 (full-length receptors) or through TOPO-reaction using the pENTR/D-TOPO vector (truncated receptors; both vectors Invitrogen). The template used to clone the ethylene receptors was either cDNA from *Arabidopsis* roots (ETR1, ERS1), or a corresponding clone kindly provided by G.E. Schaller (ETR2) and E.M. Meyerowitz (ERS2, EIN4; Hua et al. 1998). Entry clones of ETR2, ERS2, and EIN4 seem to be problematic for *E. coli*, as we observed spontaneous mutations and insertions in the cDNA sequence. The use of the *E. coli* strain CopyCutter™ (Epicentre) enabled us to amplify and clone these receptor genes. The reverse primers contained no stop codon to enable C-terminal fusions. Sequences of forward and reverse primers could be sent upon request. Construction of the truncated ethylene receptors was performed using the full-length Entry clones as template, appropriate reverse primer and the following forward primer: ΔTM-ETR1 (deletion aa1-121) 5'-CACC-ATGTTGAAAA-TAAAGCTGCTGAGCTC, ΔTM-ERS1 (aa1-121) 5'-CACC-ATGCT-CAAGAAGAAAGCTGATGAGTTAG, 5'-ΔTM-ETR2 (aa1-150) 5'-CACC-ATGCTTAAGAAGAAAGCTCATGAGC, ΔTM-ERS2 (aa1-153) 5'-CACC-ATGTTGAGTAAGAAGACCAGAGAGCTTG, ΔTM-EIN4 (aa1-144) 5'-CACC-ATGTTGAAGCAGAATGTGTTGGAGC ΔTM/GAF-ERS1 (aa1-316) 5'-attB1-TAATGCACGCTCGTGACC-AGCTTATG. All clones were carefully verified via restriction analysis and sequencing (GATC Biotech) prior to LR-reaction. For creation of the Destination clones, see below.

Agrobacterium Infiltration of *Nicotiana benthamiana* Leaves

The binary vectors for expression of the GFP/RFP fusion proteins under the control of 35S promoter were constructed via LR-reaction using the corresponding Entry clones. The

full-length ethylene receptors were cloned into the destination vectors pMDC83 (Curtis and Grossniklaus, 2003) and pB7RWG2.0 (Karimi et al., 2002) and the truncated versions into pH7FWG2.0 (Karimi et al., 2002). ER marker protein was constructed via in-frame GFP insertion into the *Arabidopsis* ORF At2g31710 (genomic DNA fragment containing promoter region) in the binary vector pTkan+ (pPZP212 derivative, Hajdukiewicz et al., 1994). Endoplasmic reticulum localization of the fusion protein was proved by immunogold labeling and electron microscopy (K. Schumacher and Y.D. Stierhof, personal communication). BRI1 plasmalemma marker (Friedrichsen et al., 2000) was kindly provided by K. Schumacher. The p19 protein from tomato bushy stunt virus cloned in pBIN61 (Voinnet et al., 2000) was used to suppress gene silencing. All vectors were transformed in *Agrobacterium tumefaciens* strain GV3101 pMP90 and prior infiltration resuspended in AS-medium (10 mM MgCl₂, 150 μM acetosyringone and 10 mM MES pH 5.7) to OD₆₀₀ 0.8. Corresponding *Agrobacterium* strains containing the GFP/RFP constructs and the p19 silencing plasmid were mixed 1:1:1 and co-infiltrated into leaves of 2–4 week old *N. benthamiana* plants, as described in Voinnet et al. (2003) and Witte et al. (2004). Abaxial epidermis of infiltrated tobacco leaves was assayed for fluorescence by confocal laser-scanning microscopy 2–3 d post infiltration.

Microscope Image Acquisition

Confocal laser-scanning microscopy (CLSM) was performed using a Leica TCS SP2 confocal microscope (Leica Microsystems GmbH). All CLSM images were obtained using Leica Confocal Software and the HCX PL APO 63×/1.2 W CORR water-immersion objective. GFP and RFP channels were acquired by simultaneous scanning using 488-/568-nm laser lines for excitation; signals were detected between 500 and 530 nm for GFP and 590 and 630 nm for RFP. Images were processed using Leica Confocal Software (Leica Microsystems GmbH) and Adobe Photoshop 9.0. Microscopy of histochemically stained primary roots was carried out on a Zeiss AxioPhot microscope (Carl Zeiss AG) equipped with an AxioCam HR CCD camera using a 10/0.3 objective. Images were acquired using AxioVision 3.1 software (Carl Zeiss AG) and processed in Adobe Illustrator 12.0.1.

Semi-quantitative RT-PCR

Total RNA from corresponding tissues and developmental stages of *A. thaliana* was isolated using RNeasyTM (Ambion) and genomic DNA was removed using TURBO DNA-freeTM (Ambion). Subsequently, 1.5 μg of total RNA was reverse transcribed using oligo-dT primer with SuperScriptTM III Reverse Transcriptase (Invitrogen) and resulting cDNA was used as a template for PCR with HotStart Taq polymerase (Genaxxon). PCR products were separated via agarose gel electrophoresis after a different number of PCR cycles for comparison with ACTIN2 at non-saturating conditions as described in Horák et al. (2003). The sequences of the primers were as follows: ACT2 detF 5'-CTGCTCAATCTCATCTTCTTCC; ACT2 detR 5'-GACCTGCTCATCATACTCG; ETR1 detF 5'-TGTCACCAAGTCA-

GACACACGA; ETR1 detR 5'-TCATGGGACACAACCTCGG AG; ERS1 detF 5'-CACTAATCAGCGACGTTCTGGA; ERS1 detR 5'-GCCCCACAAACCGTTTACAG; ETR2 detF 5'-TCGTACGATCCATGAAGCAGC; ETR2 detR 5'-ATGGTCTCCGGTGAACCATC; ERS2 detF 5'-GGGTTGCTTCCTTGATACTCA; ERS2 detR 5'-TCGATCGCCGAGTTGA; EIN4 detF 5'-ACGTTCCAGCTACATTCCTTG; EIN4 detR 5'-ATACTCTGTGTTGTCCATGCGA.

Generation of the *ETR1* and *ERS1* Promoter:*uidA* Fusions and Histochemical Staining

Promoter DNA fragments were amplified from *A. thaliana* genomic DNA using PhusionTM polymerase (Finnzymes) and the primers ETR1pF 5'-attB1-TTACTTGTGGACCAGTGTGAGC, ETR1pR 5'-attB2-GTTGCGGTTCAATACAATTGC, ERS1pF 5'-attB1-CCAACGGTGAATGCAGCA and ERS1pR 5'-attB2-GCATGCGTCTCAAAACAATCG. Promoter DNA fragments were cloned into pDONR201 through BP-reaction and verified by sequencing. The ETR1 promoter fragment comprises the region 2201 bp upstream of the start codon, including the first 30 bp of the ETR1 coding sequence and the ERS1 promoter, and the region 1811 bp upstream of the start codon, including 33 bp of the ERS1 coding sequence. Both promoters were translationally fused to *uidA* reporter gene via LR-reaction with the destination vector pMDC163 (Curtis and Grossniklaus, 2003). Plasmids were transformed in *Agrobacterium tumefaciens* strain GV3101 pMP90 and *Arabidopsis* plants were transformed via the flower-dipping method. Transformed seedlings were selected through hygromycin-resistance (see Plant material). Primary roots of light-grown seedlings, etiolated seedlings, 30 d old leaves and flowers from several independent transgenic lines (T2) were tested for GUS activity using histochemical staining as described previously in Friml et al. (2003).

Mating-Based Split-Ubiquitin System (mbSUS)

The full-length ethylene receptors were cloned via LR-reaction of the corresponding entry-clones into pMetYC-DEST and the truncated versions into pNX32-DEST and pXN22-DEST (Grefen et al., 2007). The Destination clones were transformed into the haploid yeast strains THY.AP4 (pMetYC-DEST clones) and THY.AP5 (pNX32-DEST, pXN22-DEST), respectively (Obrdlík et al., 2004). After 3 d on vector-selective media, single colonies of each clone were harvested and analyzed by western blot to guarantee correct expression of the fusion proteins (for a detailed protocol, see Grefen et al., 2007). Positive haploid yeast clones were inoculated to an OD₆₀₀ of 3.0–5.0 in vector-selective media, harvested and resuspended in an appropriate volume of YPD. The mating was performed by mixing the haploid yeast, dropping 4 ml on solid YPD media and incubating for up to 16 h at 28°C. Using a replicator stamp, the mated colonies were transferred on vector-selective media and incubated for 3 d. Colonies were then harvested, inoculated in liquid, vector-selective media and incubated overnight at 28°C. All clones were diluted to yield the same starting OD₆₀₀ and hence dropped on vector- and interaction-selective media. Starting on day 3, the growth was monitored.

SUPPLEMENTARY DATA

Supplementary Data are available at www.mplant.oxfordjournals.org

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