Monitoring Ethylene in Plants: Genetically Encoded Reporters and Biosensors

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1. Introduction

1.1. The Hormone Ethylene

Ethylene is a plant hormone best known for its key role in fruit ripening. Being the smallest among classical plant hormones, it possesses the simplest structure: ethylene is an olefin made of two carbons (C2H4) (reviewed by Ecker[1] and Wang et al.[2]). Due to its gaseous nature, ethylene moves freely between neighboring cells by diffusion across the membranes and does not require specific transporter proteins.[3] Despite its small size, ethylene is a potent growth regulator involved in every stage of the plant’s life, from seed germination to tissue senescence, and controls multiple aspects of plant physiology, from organ abscission to defense responses.[4,5] Historically, humans have been trying to manipulate the levels and responses to this hormone, starting with the ancient Egyptians and Chinese, who intentionally damaged fruits to stimulate ethylene production, to modern agriculture, where inhibitors of ethylene such as 1-methylcyclopropene are routinely used to dramatically extend the shelf life of fruits and vegetables (reviewed by Golding and Singh[11]).[6–11] In the past 40 years, molecular mechanisms of ethylene biosynthesis, perception, and signaling have been explored. The characterization of the first ethylene biosynthetic enzyme in 1979 was followed by the identification of the corresponding mRNA ten years later. Similarly, the isolation of the first ethylene receptor mutant in 1988 was ensued by the positional mapping and cloning of the causal receptor gene in 1993.[12–15] This manuscript focuses on the different approaches employed by plant biologists to monitor ethylene biosynthesis and action in plants, with a special emphasis made on genetically encoded sensors. We start by presenting the current state of knowledge of ethylene biosynthesis and signaling in Arabidopsis, then talk about hormone detection methods in plants, review the different types of reporters and biosensors, and describe the tools currently available for monitoring ethylene. We conclude with a discussion on the potential designs of next-generation ethylene biosensors that the ethylene community could potentially develop in the near future.

1.2. Biosynthesis

Ethylene is synthesized in the cytoplasm of plant cells in a three-step reaction (Figure 1).[16,17] First, the amino acid methionine is converted into S-adenosyl-methionine (SAM) by SAM synthetase (SAMS). Next, SAM is metabolized to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACS synthase (ACS). This intermediate can be actively transported to target cells or can be stored or deactivated by conjugation to make jasmonyl-ACC, malonyl-ACC, or γ-glutamyl-ACC (reviewed by Van de Poel and Van Der Straten[18]). The final step in ethylene biosynthesis is the oxidation of ACC by ACC oxidase (ACO) to produce the active hormone that is then perceived by the same cell or diffuses to neighboring cells and beyond. It is unknown if ethylene can be stored, conjugated, or deactivated.[3,19]

1.3. Perception and Signaling

Ethylene is perceived by ER-localized receptors that harbor homology to bacterial two-component histidine kinases.[19] In Arabidopsis, there are five ethylene receptors, ETHYLENE RESPONSE1 (ETR1), ETR2, ETHYLENE RESPONSE SENSOR1 (ERS1), ERS2, and ETHYLENE...
INSENSITIVE4 (EIN4) (reviewed by Gallie[20]). The binding of ethylene to the receptors is coordinated by copper ions that are delivered by the Golgi-localized protein RESPONSIVE-TO-ANTAGONIST1 (RAN1).[21,22] Genetically downstream of ethylene to the receptors is coordinated by copper ions and inhibition of EIN2 (Figure 2a), with the phosphorylated EIN2 targeted for degradation by two F-box proteins, EIN2 TARGETING PROTEIN1 (ETP1) and ETP2.[28–30] In the presence of ethylene, the receptors bind the hormone and undergo a conformational change that releases and inactivates CTR1, concomitantly ceasing the phosphorylation and inhibition of EIN2 (Figure 2b).[31,32] Thus, in ethylene, the unphosphorylated C-terminus of EIN2 is cleaved by an unknown protease and the soluble C-terminus migrates away from the ER to 1) the nucleus where EIN2 potentiates transcriptional master-regulators EIN3 and EIN3-LIKE1 (EIL1) to trigger transcriptional responses to ethylene, and 2) to cytoplasmic P-bodies where the EIN2 C-terminus directly or indirectly regulates translation of a small subset of ethylene-responsive mRNAs.[28,32–37]

In the nucleus, the levels of EIN3 and EIL1 are controlled by F-box proteins EIN3-BINDING F-BOX PROTEIN1 (EBF1) and EBF2 that in the absence of ethylene target these transcription factors for ubiquitin-mediated proteasomal degradation (Figure 2a).[38–40] In the presence of ethylene, the EBF proteins are themselves destabilized via a proteasome-dependent mechanism and turned over, leading to the accumulation of EIN3/EIL1 and activation of the transcriptional ethylene responses (Figure 2b).[41,42] EIN3/EILs recognize specific sequences, known as the ethylene binding site (EBS), in the promoters of their target genes.[43] Among the genes directly regulated by EIN3/EIL1 are second-tier transcription factors, including multiple members of the ETHYLENE RESPONSE FACTOR (ERF) and ETHYLENE RESPONSE DNA-BINDING FACTOR (EDF)/TEMPRANILLO (TEM) families, that [once translated] bind to their respective sequences (such as the GCC box in the case of ERFs) in the promoters of third-tier genes, amplifying the ethylene-triggered cascade of ethylene responses.[42–44]

In the cytoplasm, in the presence of ethylene, the EIN2 C-terminus targets a select group of transcripts, including EBF1 and EBF2 mRNAs, to P-bodies and represses transcript translation (Figure 2b).[16,17] Reduced translation of EBFs results in the stabilization of EIN3 and EILs and reinforces ethylene signaling.[38–40] It is currently unknown whether the binding of EIN2 to the EBF transcripts (direct or indirect) takes place in the cytoplasm or if the EIN2/EBF-containing ribonucleoprotein complex initially assembles in the nucleus and later translates to the cytoplasmic P-bodies. When the ethylene signal ceases, the cytoplasmic EBF transcripts are released from the P-bodies and become rapidly translated, leading to a buildup of EBF proteins and consequent turnover of EIN3/EIL1, thus stopping ethylene responses.[16,17]

1.4. Regulation

Ethylene production and signaling are subject to complex regulation that involves feedback-controlled transcriptional loops, translational regulation, and post-translational modifications.[16,17,45] In ethylene biosynthesis, ACS proteins are believed to catalyze the rate-limiting step of the pathway, whereas ACO proteins are thought to be predominantly constitutively made in most vegetative tissues.[46] Nonetheless, examples of transcriptional regulation by various endogenous and exogenous stimuli have been reported not only for ACSs, but also for some ACOs, suggesting that under some conditions ethylene evolution is controlled not at the step of ACSs, but at the level of ACOs (reviewed by Rudu[47]). However, the predominant view in the field is that ACSs are the primary targets of regulation.[48] In fact, multiple isoforms of ACS are differentially transcriptionally induced or repressed by various environmental and developmental signals.[10,49,50] Furthermore,
the stability of some ACS proteins is controlled at a post-translational level, with kinase-mediated phosphorylation of ACS leading to protein stabilization and higher ethylene production and, conversely, phosphatase-mediated dephosphorylation resulting in ACS protein ubiquitination, proteasomal degradation and, consequently, inhibition of ethylene biosynthesis.[48]

Several of the ethylene signaling components are themselves regulated by ethylene. As described above, the positive regulators of the ethylene pathway, EIN2 and EIN3/EIL1, are stabilized in the presence of ethylene at the protein level.[51] In contrast, negative regulators, F-box proteins EBF1 and EBF2, as well as one of the ethylene receptors, ETR2, are turned over more rapidly in ethylene in a proteasome-dependent manner.[40,52–54]

Two additional F-box proteins that target EIN2 for degradation, ETP1 and ETP2, are also destabilized by ethylene, allowing for EIN2 accumulation.[51] Cumulatively, these post-translational changes are expected to reinforce ethylene signaling.

At the transcriptional level, several of the negative regulators of the pathway, including ETR2, ERS1, ERS2, CTR1, EBF1, and EBF2, are transcriptionally induced by ethylene, a phenomenon that may lead to the attenuation of ethylene signaling should the transcripts become efficiently translated.[42] However, at least in the case of EBF1 and EBF2, translation of their mRNAs in ethylene is impaired, suggesting that this EIN2-dependent translation inhibition mechanism may serve to prevent premature synthesis of these F-box proteins to avoid untimely translation of EIN3/EIL1 mRNAs to the P-bodies and inhibits their translation preventing further F-box production; in the nucleus, it triggers degradation of the EBF1/2 proteins and potentiates transcription factors EIN3/EIL1 (dashed lines). Stabilization of EIN3/EIL1 results in the activation of ethylene responses.
dampening of the ethylene response, yet enable rapid synthesis of the EBFs, trigger degradation of EIN3/EILs and stop transcriptional responses once ethylene is withdrawn.\textsuperscript{[16,37]} It is noteworthy that the major positive regulators of the pathway (EIN2 and EIN3/EIL1) are not transcriptionally regulated by ethylene, suggesting that in these cases the post-translational mechanisms of regulation prevail.

1.5. Hormone Detection Methods

To assay ethylene in plants, several approaches are commonly employed by the plant community: analytical quantification using specialized instruments, phenotypic and molecular assays done on whole plants or individual organs or tissues, and genetically encoded biosensors. Historically, the detection of ethylene has been viewed as important given the central role of this hormone in plant senescence and fruit ripening (recently reviewed by Iqbal et al.\textsuperscript{[53]}). Monitoring and controlling ethylene accumulation is, therefore, essential to preventing fruit and vegetable spoilage. Hence, several ethylene sensors (i.e., electrochemical sensors or laser-based sensors) and analytical devices (gas chromatography) have been developed or adapted in the last decades to detect and quantify ethylene’s presence in air samples, both in laboratory settings and in fruit and vegetable storage facilities.\textsuperscript{[56–61]}

Phenotypic assays have been extensively used in research laboratories to study the role of ethylene in plant physiology and development, to investigate the contribution of this hormone to the phenotypes of one’s favorite mutants, and to identify molecular players of the ethylene biosynthesis and signaling pathway.\textsuperscript{[62]} The triple response assay carried out in young seedlings germinated in the dark in the presence of ethylene or its precursor ACC is a widely used method for screening for ethylene-response defects and to illuminate molecular mechanisms underlying ethylene perception and signaling (reviewed by Merchante and Stepanova\textsuperscript{[63]}). Wild-type plants in this assay display short and thick hypocotyls, exaggerated apical hooks and short roots, whereas various mutants with defects in the perception, signaling or response to ethylene or in uptake of ACC show complete or partial ethylene insensitivity, e.g. long roots or reduced apical hooks.\textsuperscript{[61]}

Besides monitoring plant morphology, molecular tools are often employed to assay expression of ethylene-regulated genes. The expectation is that if a plant has a defect in ethylene biosynthesis, perception, signaling or response, the transcriptome of this plant line will be affected in a manner that ethylene-related genes will be over- or under-represented among the genes differentially regulated relative to wild-type plants. While in the past Northern blots with ethylene marker genes (e.g., basic chitinase, \textit{ERF1} and other \textit{ERFs}, etc.), in situ hybridizations, and microarrays were the primary methods of choice, these days, qRT-PCR and RNA-seq are the predominant technologies scientists rely upon to evaluate ethylene responses.\textsuperscript{[64–66]} There is a wealth of prior knowledge on ethylene-regulated genes, especially in model organisms, which enables investigators to compare their datasets with previously published data.

The last method commonly used in plant biology to evaluate ethylene is genetically encoded reporters and biosensors. This term refers to a collection of stably or transiently expressed DNA constructs that allow researchers to visualize and quantify ligand (in this case, ethylene) biosynthesis, binding, signaling, or response. These types of sensors consist of a sensory module that responds to the ligand of interest (herein, ethylene) fused to a reporter gene that produces an easy-to-read signal.\textsuperscript{[67–69]}

Below we describe the three main types of genetically encoded biosensors commonly used in the study of phytohormones.

1.6. Reporters and Biosensors

1) Receptor-based ratiometric Föster resonance energy transfer (FRET) sensors rely on a pair of fluorescent proteins, a donor, and an acceptor, that flank a sensory ligand-binding module. When the ligand is detected, a conformational change in the sensory module alters the distance and/or the orientation of the two FRET components, resulting in a ratiometric shift in the emission spectra of the fluorescent proteins in the FRET pair.\textsuperscript{[57,58,67,70]} The closer the two fluorescent proteins are brought together, the greater is the energy transfer, and the more prevalent is the emission of the acceptor protein relative to that of the donor. Up to date, only two hormones have been studied using this method: abscisic acid (ABACUS1 and ABAleon2) and gibberellins (GPS1), whereas ratiometric ethylene sensors are yet to be developed (see below).\textsuperscript{[70–72]}

2) Degradation-based reporters take advantage of the protein degradation module of a core hormone signaling pathway, such as that triggered by auxin to inactivate the AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) repressor proteins.\textsuperscript{[73]} In this scenario, a fluorescent (e.g., \textit{VENUS}) or luminescent (e.g., \textit{LUCIFERASE}) reporter is fused to the sensory module. The sensory module could be an entire protein (e.g., full-length IAA28) or a protein degron domain (e.g., a subdomain of IAA28, DII) that becomes ubiquitinated and degraded in response to the hormone stimulus (in this example, auxin) along with the reporter it is fused to, thus leading to the sensor turnover and disappearance of the fluorescence/luminescence signal. In the absence of the hormone, the reporter is active and is detectable via fluorescence or luminescence, whereas in the presence of the hormone, the reporter is targeted by the 26S proteasome for proteolysis and destroyed. The loss of fluorescence or luminescence is thus indicative of active signaling.\textsuperscript{[57,67,69]}

Degron-based sensors have been successfully employed in the study of auxin (DII-VENUS, L2min17-Luc), gibberellins (GFP-RGA), and jasmonic acid (JAS9-VENUS).\textsuperscript{[73–77]} In the ethylene field, full-length fluorescently tagged EBF1, EBF2, EIN2, EIN3, and EIL1 proteins whose stability is regulated by ethylene are available, but minimal degrons of these proteins are yet to be defined (see below).\textsuperscript{[78–80]}

3) Expression-based reporters monitor responses to the hormone of interest by measuring stimulus-inducible or repressible gene activity at the transcriptional and/or translational level. An entire hormone-regulated gene or its hormone-responsive region is fused with a reporter gene, such as fluorescent (green fluorescent protein, \textit{GFP}), luminescent (luciferase, \textit{LUC}), or colorimetric (β-glucuronidase, \textit{GUS}) markers. Hormone-responsive sequences can be naturally occurring (e.g., a full-length or partial native promoter or 3′
untranslated region (UTR)) (Figure 3a,c) or synthetic (e.g., a tandem of consensus hormone-responsive elements from the promoter or the 3′ UTR of a stimulus-regulated genes) (Figure 3b,d). Promoter–reporter fusions enable monitoring of transcriptional regulation, whereas reporter fusions to the 5′ or 3′ UTR of a gene of interest assay translational regulation. Finally, in-frame full-gene reporter fusions monitor a combination of transcriptional and post-transcriptional effects. The functionality of this last class of constructs can be tested by phenotype complementation of the corresponding mutant. This approach assures that GFP (or another reporter) does not interfere with the function of the tagged gene of interest and all regulatory sequences have been included in the fusion construct. Constructs that can fully complement the mutant can then be relied upon to infer the expression patterns of the endogenous gene of interest, including its hormone-regulated expression. The downside of using full-gene and native promoter–reporter or reporter–UTR fusions as hormone sensors is that they are usually not specific for any given hormone: they harbor an array of regulatory sequences and thus are regulated by a variety of endogenous and exogenous factors. Synthetic reporters, on the other hand, aim to reduce the diversity of regulatory elements present and often harbor a tandem of multiple repeats of a single element, such as a transcription factor binding site in the case of transcriptional regulation reporters or a translation factor binding sequence in the case of translation regulation constructs.

In the past few years, the plant community has embraced the concept of synthetic expression-based reporters and generated dozens of useful hormone sensors. Synthetic transcriptional regulation reporters are available for auxin (DR5:GUS as well as fluorescent and luminescent versions of DR5rev and DR5v2), cytokinins (TCS:GFP and TCSn:GFP), abscisic acid (6xABRE:GFP), jasmonate (p4D-47), salicylate (4xPR1:pporRFP), and ethylene (5xEBS:GUS).[79–90] On the other hand, translational regulation reporters have thus far been described only for ethylene, and both native (35Sp:GFP-3′EBF2, 35Sp:GFP-3′EBF1) and synthetic (35Sp:GFP-6xEPU) ethylene-repressible versions have been reported.[36,37] Despite the wide variety of hormone biosensors published to date, in the ethylene field only expression-based reporters have been broadly employed, alongside with phenotypic bioassays. As quantitative and phenotypic methodologies commonly utilized in ethylene research have both been reviewed recently, we chose to focus the rest of this manuscript on genetically encoded ethylene biosensors.[56,63] A majority of previously published studies that discuss plant hormone biosensors do not cover ethylene reporters.[68,69,91] With this article, we hope to fill in that gap and to describe existing biosensors readily available to ethylene researchers, as well as evaluate additional ethylene reporter designs that can be pursued in the future.

2. Reporting Ethylene in Plants

In plant biology, the sites of ethylene production are typically visualized with the help of transcriptional fusions between promoters of ethylene biosynthesis genes with GUS or GFP. Likewise, the tissues that perceive and respond to ethylene can be marked by means of whole-gene or promoter-only fusions between up- or down-regulated ethylene target genes and GFP, GUS, or LUC. Native promoter fusions were the only reporters available in the ethylene field until mid-2000s, but since then

![Figure 3. Different types of expression-based ethylene reporters.](image-url)
synthetic transcriptional reporters based on the EIN3 binding sequence have become more popular.[89,92–96] Furthermore, translational effects of ethylene can be monitored with the help of reporter-3′UTR fusions in which the 3′UTRs of an ethylene-regulated translationally repressible gene cloned downstream of the stop codon of GFP (or of another reporter) confers ethylene-repressible expression.[16,37] Alternatively, synthetic translational regulation reporters that carry tandem arrays of the cis-element sufficient for this translational regulation can be employed.[37] Translational reporters are a fairly recent development in the ethylene field and have not yet been widely adopted by the community, so transcriptional reporters continue to dominate in ethylene research.

2.1. Biosynthetic Reporters

To study when and where endogenous ethylene biosynthesis takes place and how it is regulated in the course of plant development and in response to various environmental factors, numerous studies relied upon transcriptional fusions of ACS and ACO with GUS or GFP. For example, the role of ACS2 (ACS1 in the old nomenclature) in the synthesis of ethylene during Arabidopsis seedling development was demonstrated using a promoter-GUS fusion.[97] Tsunchisaka and Theologis[49] studied the expression patterns of promoter fusions for nine Arabidopsis ACS family members with GFP and/or GUS during development and under an array of treatments (auxin, mechanical wounding, cold, heat, anoxia, and lithium ions), uncovering overlapping and unique domains of expression. Wang et al.[98] examined the regulation of ACS4, ACS5, and ACS7 by auxin, cytokinin, gibberellin, ethylene, and abscisic acid, as well as by osmotic stress, darkness, darkness plus low temperature, high temperature, wounding, drought, and anaerobic treatment, shedding light on the relative contributions of these three ACS genes in basal and stimulus-triggered ethylene biosynthesis. More recently, Arabidopsis ACS8 was implicated in copper ion-triggered ethylene biosynthesis, and promoter deletion analysis in the context of GFP reporter constructs lead to the identification of a copper-response cis-element (cuRE) in the promoter of ACS8.[99]

In species beyond Arabidopsis, transcriptional fusions of ACS genes with GUS have been characterized in tomato, mulberry, and apple, proving instrumental in uncovering unique patterns of ACS gene expression and regulation, and shedding light on the role of ethylene in controlling specific aspects of these plants’ physiology.[100–102] Similarly, ACO gene promoter-GUS fusions in tomato, Nicotiana plumaginifolia, apple, melon, and peach enabled the study of the role of ACO genes in ethylene biosynthesis during fruit ripening, plant development, and defense.[93,103–106] In Arabidopsis, recent studies of ACO1p:GUS fusions implicated this gene in hormone-mediated regulation of ethylene biosynthesis, with brassinosteroids, auxin, ethylene, and its precursor ACC all promoting ACO1 expression in a tissue-specific manner, and ethylene inhibitor aminoethoxyvinylglycine and gibberellic acid inhibiting ACO1 promoter activity in non-overlapping subdomains of the root.[100] ACO1 promoter deletion analyses in the context of GUS reporters pinpointed two E-box motifs critical for the light-mediated regulation of ACO1 gene expression.[106] Taken together, the employment of ACS and ACO transcriptional reporters enabled a much greater level of resolution (as compared to Northern blots, qRT–PCR and other classical transcriptomic methods) and implicated the tissue-specific regulation of individual ACS and ACO ethylene biosynthesis genes not only in normal plant development, but also in responses to various stimuli.

2.2. Transcriptional Regulation Reporters

Given the central role of ethylene in pathogen responses (reviewed in Broekaert et al.[107]), some of the first reporters made back in the late 80s and early 90s and utilized in the ethylene field were GUS fusions with the promoters of pathogenesis-related genes, several of which are induced by pathogen attack. For example, tobacco BASIC-TYPE PR-1 gene PRB-1b, bean and tobacco BASIC CHITINASE genes CHSB and CHN48 (Figure 3a), and a tobacco BASIC BETA-1,3-GLUCANASE gene all show ethylene-inducible behavior.[41,95,108–110] Bean CH5B also works in the heterologous plant system, Arabidopsis.[111] Likewise, given the key involvement of ethylene in the ripening of climacteric fruits, promoters of tomato ripening-related genes were leveraged to build transcriptional sensors responsive to ethylene produced in the course of fruit ripening.[112] For example, a LUC gene fusion with the promoter of the tomato E4 gene (Figure 3a) displays ethylene-regulated expression and linker scanning approaches in transient reporter assays have been instrumental in narrowing down the region of the E4 promoter required for its ethylene-inducible behavior.[113,114] Similarly, promoter deletions of another tomato ripening gene, E8, generated in the context of a genomic DNA construct were used to investigate ethylene-regulated behavior of E8 reporters by tagging the gene with a piece of lambda phage DNA and relying on Northern blots to detect the tagged transcripts.[115] Further studies on the E8 promoter resulted in the identification of a ~430 bp distal fragment that is sufficient to confer ethylene-inducible behavior to the 35S minimal promoter-driven LUC gene in unripe fruits treated with ethylene.[116]

Over the years, high-throughput transcriptomic studies have uncovered hundreds of additional ethylene-regulated genes in Arabidopsis and other species, providing numerous candidates for transcriptional reporter fusions.[42,117–124] In the course of functional characterization of some of these genes, reporters have indeed been made, but none of them are expected to be exclusively ethylene-regulated and thus are not optimal for ethylene monitoring. The list of ethylene-regulated genes contains several of the core components of the ethylene signaling pathway that are direct targets of EIN3, including ERS1, ERS2, ETR2, CTR1, EBF1, and EBF2 in Arabidopsis.[42] Of these promoters, fusions with GUS were reported for ETR1 and ERS1, that showed partially overlapping domains of expression in Arabidopsis but have not been examined for ethylene inducibility.[125] An equivalent promoter fusion for the tomato ERS1 ortholog, NEVER-RIPE (NR), was also reported, but its ethylene-regulated behavior was not tested.[113] On the other hand, a similar GUS fusion of an orchid ERS1 promoter showed ethylene-inducible expression, but the reporter was
also inducible by several sugars (glucose, maltose, fructose, and lactose) and repressible by mannitol, hormones (auxin, cytokinin and abscisic acid), and several abiotic stress factors (heat, wounding, high salt, drought, and flooding). The Arabidopsis ETR2 promoter fused to GUS showed trichome-enriched expression, but again, its ethylene-mediated regulated was not analyzed. A CTR1 promoter fusion with GUS was reported under the name T116 and showed inducibility by ACC and ethylene. Finally, transcriptional fusions of the EBF1 and EBF2 promoters with GUS have both been published, but only EBF2 showed ACC-inducible expression. In the same study, an LUC version of the EBF2 reporter was also made and tested in transient assays with and without EIN3. As expected, a co-transfection with EIN3 could induce LUC activity of the wild-type EBF2p:LUC reporter construct, but not that of the mutant version in which the EIN3 binding site was mutated.

Despite the ethylene-regulated and, in some cases, EIN3-dependent expression of the aforementioned transcriptional reporters for the core ethylene signaling components, neither of these native promoter fusions can be considered as reliable ethylene reporters as, based on publicly available transcriptomic data, these genes (like any other ethylene-regulated genes) are controlled by multiple factors besides ethylene itself.

2.3. Synthetic Transcriptional Reporters

The simple solution to the problem of native promoters (that collect the input of many transcription factors regulated by many different stimuli and, hence, are not ethylene-specific) is to isolate the binding sites of interest (in our case, ethylene-related) from the native promoters and stack them together, getting rid of all irrelevant sequences. Typical synthetic transcriptional reporters contain an array of identical or similar sequences recognized by a single transcription factor family (e.g., EIN3/EIL1 binding sites (EBS) for an ethylene reporter, or ARF binding sequence for an auxin reporter) upstream of a minimal promoter (that provides core sequences like the TATA box for the recruitment of general transcription factors and RNA polymerase II) driving expression of a reporter of interest, such as GFP, LUC, or GUS. Such reporters are expected to be highly specific to the process of interest, as only one family of transcription factors can regulate its expression. Using this logic, four versions of the EBS reporter that monitor EIN3/EIL1 activity have been made. The 5xEBS:GUS reporter harbors a tandem of five copies of the EIN3-binding site from the promoter of one of the immediate targets of EIN3, EDF1, fused to the minimal 35S promoter followed by the open reading frame of GUS (Figure 3b). Its derivative, 5xEBS:Luc, in which LUC replaces GUS has also been reported. The 4xEBS:LUC reporter carries four repeats of the EBS from the promoter of ERF1 fused to the 35S minimal promoter and LUC gene (Figure 3b). Finally, the fourth version of EBS, known as 2EBS-S10-36, carries 36-nucleotide-long inverted tail-to-tail dual synthetic repeats that have been optimized in vitro for EIN3 binding in terms of both spacing and orientation of the EBS sequences. While stable transgenic lines exist for the GUS and LUC versions of 5xEBS, only transient expression vectors have been described for the 4xEBS and 2EBS-S10-36 versions. Of the four reporters, only 5xEBS:GUS is widely adopted by the community and over the years has proven instrumental to implicating ethylene in a wide variety of processes. For example, 5xEBS:GUS is induced in Arabidopsis by senescence, by interactions with a root-knot nematode Meloidogyne hapla and a soybean cyst nematode Heterodera glycines, by abiotic factors such as aluminum, iron, cadmium, chromium, mitochondrial translation inhibitor doxycycline, elevated or low nitrate doses, ammonia, boron deficiency, alkaline pH, and high salt.

Besides Arabidopsis, 5xEBS:GUS was also employed in tomato to investigate ethylene responses in ripening fruits and in a hemiparasitic plant Triphysaria versicolor to visualize ethylene responses in early haustorium development upon treatment with a haustorium-inducing factor, DMBOQ. Despite its wide-spread use, the 5xEBS:GUS reporter has limited sensitivity, and no fluorescent reporter version has been published, so there is clearly some room for further optimization of this biosensor.

2.4. Translational Regulation Reporters

Besides affecting transcription of hundreds of genes, ethylene has been shown to repress translation of a handful of transcripts (including EBF1 and EBF2) in an EIN2-dependent and EIN3/EIL1-independent manner. Two groups in parallel described the ability of ethylene to modulate translation in a gene-specific fashion and demonstrated that the cis-elements required and sufficient for the translational regulation of EBF1 and EBF2 are confined to the 3′ UTRs of these genes. In fact, the 3′ UTRs cloned downstream of a reporter could confer ethylene-mediated translational repression. Transgenic plants harboring a 35S-promoter-driven GFP followed by the 3′UTR of either EBF1 or EBF2 made less GFP protein (but not of GFP mRNA) and showed less fluorescence in ethylene than in air, indicating that the translational inhibition by ethylene is mediated by these 3′ UTRs. Thus, these constructs represent effective translation regulation reporters for monitoring ethylene activity at the EIN2 level, although signals other than ethylene may also control their expression. In the original studies, these 3′ UTR-based translation regulation constructs/transgenes were referred to as 35SpGFP:3′EBF1 (or G1U) for the EBF1 3′ UTR and 35SpGFP:3′EBF2 (or G2U) for the EBF2 3′ UTR (Figure 3c).

The EIN2-dependent translational regulation mechanism uncovered by Li et al. and Merchant et al. is essential for normal responses to ethylene, as removing or replacing the EBF 3′ UTR with a generic terminator sequence or with a mutated 3′ UTR from which all U-rich motifs were deleted leads to ethylene insensitivity of the transgenic plants. Bioinformatic analysis of the 3′ UTRs of the genes translationally repressible by ethylene identified a poly(U) cis-element that is enriched in this subset of genes and is present multiple times in both the EBF1 and EBF2 3′ UTRs. Sequence deletion analysis that eliminated poly(U) sequences from the 3′ UTR of EBF1 abolished the ethylene-repressible behavior of the construct, whereas a synthetic tandem of six copies of poly(U) conferred ethylene-repressible expression to a GFP reporter. The latter construct, named by the authors 6xEPU, represents the first
Figure 4. A hypothetical FRET-based ratiometric ethylene sensor. The reporter consists of three transmembrane alpha-helices (gray cylinders) of an ethylene receptor's ethylene binding domain (EBD), as well as two fluorescent proteins, such as CFP (blue cylinders) and YFP (yellow cylinders), fused to the EBD on same side of the ER membrane (in this example, on the cytoplasmic side). The EBD coordinates copper ions (brown circles) and forms a homodimer through disulfide bridges (S–S) at the N-terminal end. a) In the absence of ethylene, the CFP and YFP proteins are positioned too far from one another, so that upon illumination of the sensor at the CFP absorption wavelength (blue lightening), little or no energy is transferred to YFP (black arrow), and the light is emitted predominantly in the CFP emission range (blue halo). b) Upon binding of ethylene (green oval), the conformation of the EBD is changed in a way that the two fluorescent proteins are brought close to one another, enabling energy transfer from CFP to YFP (black arrows) and leading to the emission predominantly in the YFP spectrum (yellow halo).

3. New Frontiers for Ethylene Detection

Despite the well-documented utility of the aforementioned reporters in ethylene research, the array of genetically encoded sensors currently available in the ethylene field is not as diverse as the toolsets generated for some other plant hormones. The ethylene community should therefore strive to build new ethylene sensors, such as those that measure direct binding of ethylene, visualize reporter protein degradation/stabilization in the absence/presence of ethylene, and/or monitor EIN2 C-end translocation into the nucleus.

None of the ethylene reporters developed to date monitor ethylene binding, but having a FRET-based ratiometric fluorescent ethylene sensor (Figure 4) analogous to ABACUS or ABAleons for abscisic acid or GPS1 for gibberellins would offer rapid, direct means of measuring ethylene in plant tissues.[67,71,72] Given a large number of ethylene-binding proteins known in plants and cyanobacteria, it is worthwhile to investigate whether one of the two FRET components can be fused to ratiometric fluorescence shifts in minus versus plus ethylene, unless ethylene binding leads to a major conformational change in the EBD that alters the physical distance between the EBD-linked fluorescent proteins. Changes in the position of fluorescent proteins relative to one another and thus in the distance between the two upon ethylene binding will affect the intensity of FRET (the closer, the greater the FRET) (Figure 4).

While conceptually the design of ratiometric sensors is straightforward, judging from the scale of the pipeline required to develop ABACUS, this is an arduous experimental task that requires advanced synthetic biology and fluorescent microscopy skills to generate and test multiple (typically dozens of) permutations of various EBDs and FRET pairs fused via an array of linkers.[71] Another critical step in the FRET biosensor development pipeline is the optimization of a ligand binding domain through site-directed or random mutagenesis. For the EBD of the Arabidopsis ethylene receptor ETR1, an array of 41 amino acids substitution mutants have already been tested for ethylene binding, thus potentially informing the process of EBD optimization in the context of the biosensor.[150-154,157,158] It is, however, critical that the binding of the ethylene ligand to the EBD remains reversible, so that fluctuations in ethylene levels in live plants can be monitored over time as transient changes in FRET. The downsides of direct biosensors that bind the ligand with high affinity and/or irreversibly are the sequestration of the active signaling molecule and the interference with native protein interaction partners by the ligand-binding domain, phenomena that lead to the altered sensitivity of transgenic plants to the signal the sensor monitors.[71] Another potential hurdle is that ratiometric sensors have a tendency to get silenced in stably transformed plants.[71,159,160]

The second exciting route for developing a new ethylene reporter is the inclusion of an ethylene-specific destabilization domain in a constitutively expressed reporter protein, as was...
fragments that harbor only the destabilization domains of these proteins has not been reported, but may also lead to ethylene insensitivity by overwhelming the proteasome and thus sparing endogenous full-length EBFs and ETPs from degradation and, again, enhancing EIN3 and EIN2 protein turnover. To date, the identification of the minimal domains of ETPs and EBFs necessary for their destabilization in the presence of ethylene has not yet been carried out, and it is unknown if these F-box proteins self-ubiquitinate or how ethylene triggers their turnover. What has been demonstrated is that EIN2 is necessary and EIN3/EIL1 are not required for the ACC-triggered EBF destruction.\[46\]

A complementary strategy for developing an ethylene-responsive degradation-based reporter would be to visualize protein stabilization in the presence of ethylene using fluorescently tagged EIN3/EIL1 or EIN2 proteins (Figure 5b)\[28,34,38,40,164,165\]. However, expression of extra copies of these positive regulators of ethylene signaling can make plants more sensitive or constitutively responsive to ethylene and, thus, may not be ideal.\[28,35,166\] Identification of the minimal regions of EIN2 and EIN3 necessary for their respective F-box protein targeting may bypass this issue. In Arabidopsis EIN2, the most C-terminal 248 amino acids are required and sufficient for the interaction of EIN2 with ETPs, at least in a heterologous yeast system, but the minimal EIN2 domain needed for the ETP-mediated degradation has not been tested in planta.\[32\] In theory, fusions of this most-C-terminal EIN2 region (or its further truncated versions) with a fluorescent protein may provide an alternative way to visualize the ethylene-triggered loss of ETP by tracking ethylene-mediated stabilization of the EIN2-based reporter. For EIN3, mapping and narrowing of the domain sufficient for the interaction of EIN2 with ETPs has not been pursued. Only the full-length version of EIN3 is known to bind EBFs, whereas the EIN3 N-terminus is insufficient to interact or how ethylene triggers their turnover. What has been demonstrated is that EIN2 is necessary and EIN3/EIL1 are not required for the ACC-triggered EBF destruction.\[40\]

Figure 5. A hypothetical degradation-based ethylene sensor. a) A minimal domain of the EBF1/2 or ETP1/2 protein sufficient for ubiquitination and proteasome-mediated turnover (degron, DEG domain) is fused to GFP. In the absence of ethylene, the fusion protein is stable and fluorescence is observed. In the presence of ethylene, the protein is ubiquitinated and degraded, so fluorescence fades away. b) A degron of EIN2 or EIN3 sufficient for the ETP1/2- and EBF1/2-mediated recognition and proteasomal turnover is fused to GFP. In the absence of ethylene, the fusion protein is unstable and fluorescence is not observed. In the presence of ethylene, the protein is stabilized and fluorescence is visible.

done for DII-Venus sensor for auxin.\[71\] By analogy with the auxin pathway where activation of auxin signaling results in the proteasomal degradation of negative regulators Aux/IAAs, ethylene signaling leads to the physical destruction of the negative regulators of the ethylene pathway, F-box proteins ETPs (that target EIN2) and EBFs (that target EIN3).\[38,39,51,161–164\] Visualization of fluorescently tagged ETPs and EBFs that become degraded in the presence of ethylene can in theory serve as an alternative way of monitoring ethylene signaling (Figure 5a). While to our knowledge, no ETP reporters have been characterized, for the 35S:EBF1-GFP and 35S:EBF2-GFP fusions, basal levels of endogenous ethylene were sufficient to destabilize the reporter proteins, making fluorescence in wild-type plants under control conditions undetectable, but readily observable in the presence of silver ions (that interfere with the ethylene binding) or proteasomal inhibitor MG132 (that blocks protein turnover).\[46\] However, because the expression of extra copies of ETPs and EBFs is known to compromise the sensitivity of transgenic plants to ethylene by increasing the turnover of EIN2 and EIN3, respectively, the use of full-length proteins in the reporters is not advisable.\[18,39,51\] On the other hand, overexpression of truncated and thus nonfunctional EBF and ETP

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The final approach we envision builds on the phenomenon of EIN2 C-terminus cleavage and translocation to the nucleus as a marker of ethylene signaling and aims to specifically detect the nuclear form of EIN2 (Figure 6). We suggest to constitutively express the truncated (and, hence, nonfluorescent) form of a self-assembling version of GFP (saGFP1–10β, that encodes 10 out of 11 β-strands of the GFP β-barrel) in the nucleus by fusing this incomplete GFP with a nuclear localization signal.[167–171] In parallel, we fuse the C-terminal end of a constitutively expressed full-length version of EIN2 to a short remaining fragment of saGFP (saGFP11β that encodes the 11th β-strand of the GFP β-barrel) (Figure 6a).[167–171] In plants that co-express both constructs, cleavage and translocation of the tagged EIN2 C-terminus into the nucleus in the presence of ethylene will result in the spontaneous reconstitution of the full-length saGFP and, hence, nuclear fluorescence (Figure 6b).

In the absence of ethylene, no fluorescence is expected due to the physical segregation of the two parts of saGFP in different compartments (saGFP1–10β in the nucleus and saGFP11β on the cytoplasmic side of the ER) (Figure 6a). The possible hurdles of this approach are the low (and thus difficult-to-detect) levels of EIN2 and the likely hyperactivation of ethylene responses upon EIN2 C-terminus translocation to the nucleus if the EIN2 construct is driven by a strong constitutive promoter like 35S.[18,29] As with other aforementioned approaches, identification of a minimal domain of EIN2 that can migrate to the nucleus in response to ethylene but fails to potentiate EIN3 is necessary and would solve the ethylene hypersensitivity issue in lines constitutively expressing EIN2.

In summary, several complementary strategies can be pursued to develop novel biosensors for ethylene, but each approach is inherently risky and neither is fail proof. Perhaps, advancing on multiple fronts in parallel, as well as optimizing existing synthetic transcriptional (5xEBS) and translational (6xEPU) reporters to increase their sensitivity (e.g., by testing other related sequence variants, changing repeat copy number, orientation and spacing) and to diversify outputs (to include different fluorescent proteins, colorimetric markers, and multiple versions of luciferase) is probably the best way forward.[17,18]

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Conflict of Interest

The authors declare no conflict of interest.

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biosensors, ethylene, hormones, reporters, signaling


